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**PROPERTIES OF TWO DNA HELICASES OF HUMAN
CELLS**

Alexander Ochem

**A Thesis Submitted in Fulfilment of the Requirements of
The Open University for the degree of Doctor of Philosophy**

April 1999

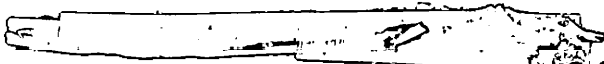
Enzymology

**International Centre For Genetic Engineering and
Biotechnology**

Trieste

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*To my beloved wife and children
whose support, encouragement and patience
never wavered all along*

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ABSTRACT

DNA helicases are ubiquitous, non-specific dsDNA unwinding enzymes involved in all aspects of DNA metabolism. In the present work, I describe the properties of two DNA helicases of HeLa cells: those of a novel enzyme, human DNA helicase VII (HDH VII), and those of the separate subunits of human DNA helicase II.

HDH VII possesses the highest specific activity among all the helicases, so far, extracted and characterized from HeLa cells, and this activity is further stimulated nearly a hundred-fold by hRPA. However, its abundance in the cell is very low since only 110 μ g of pure protein could be recovered from 150 grams of cultured cells. The estimated molecular weight of HDH VII in its native form is close to 90-kDa whereas SDS-PAGE analysis reveals two unequal, closely-migrating bands in the region of 32-kDa, suggesting that the protein may have a hetero-trimeric conformation. The enzyme exhibits a double polarity of translocation: a property that distinguishes it from all characterized eukaryotic DNA helicases. In spite of its high specific activity, HDH VII shows a relatively low processivity even in the presence of hRPA and cannot unwind duplexes longer than 17 base pairs.

The Ku antigen consists of two subunits of 70 and 83 kDa, separable from each other only by electrophoresis under denaturing conditions, and is endowed with both duplex DNA end-binding capacity and helicase activity. I have dissected the *in vitro* activities of the Ku molecule, and I report that whereas the DNA end-binding property remains a prerogative of the heterodimer, the helicase activity of the Ku molecule resides exclusively in the 70 kDa subunit, and that the helicase activity of the Ku heterodimer is stimulated upon phosphorylation by the DNA-dependent protein kinase DNA-PK.

1. 1. INTRODUCTION

1.1. Helicases and the DNA Molecule

In all organisms, the genetic information is encoded in the nucleotide sequence of the DNA molecule. Double stranded DNA (ds DNA) is the thermodynamically stable form of the molecule and this stability is guaranteed, among other things, by the inter-strand hydrogen bonds which result from the specific A-T and G-C nucleotide pairings throughout the DNA molecule. In fact, any erroneous pairing of these nucleotides gives rise to a mismatch which, if not corrected, could have negative consequences for the organism.

However, in order to accomplish most of its *in vivo* functions such as replication, recombination and repair the ds DNA needs to be present, at least transiently in the single stranded (ss DNA) form (1). DNA helicases are a special class of enzymes that unwind ds DNA, in an energy-dependent manner, in virtually all the cellular processes that necessitate ssDNA as template or as reaction intermediates *in vivo*. The energy required in the DNA unwinding catalyzed by helicases is derived from the hydrolysis of the γ -phosphate of a ribonucleoside or deoxy-ribonucleoside 5'-triphosphate in the presence of Mg^{2+} or, sometimes, some other divalent cations (1). As a direct consequence of this necessity to hydrolyze NTPs, all DNA helicases are also DNA-dependent nucleoside 5'-triphosphatases (NTPases). The use of energy to unwind and to translocate along the DNA molecule constitutes the principal characteristic features common to all known helicases and distinguishes them from other proteins that may unwind the DNA double helix passively without NTP hydrolysis (2,3). All cells contain variable quantities of different DNA helicase species. This fact obviously points to the multiplicity of *in vivo* DNA metabolic processes in which these ubiquitous enzymes play essential roles and to the different mechanisms of action employed by the different molecules (4).

Historically, the first DNA helicase was isolated in 1976 from *Escherichia coli* (5,6). Since then, increased awareness of the importance of these enzymes has led to the isolation and characterization of, at least, twelve other helicases from the same organism (7-11) as well as from other cells (12). Today we know that some inherited genetic disorders such as Bloom's syndrome, Werner's syndrome, and certain predispositions to skin cancers, that result from defects in the cellular mechanisms for the repair of DNA damage, are direct consequences of mutations in helicase genes (13-15). Interest has consequently grown in the study of DNA helicases and a large number of these enzymes have also been characterized from a wide variety of sources such as viruses (16) and eukaryotic systems including human cells (17-19). Helicases interact with ssDNA (some helicases also interact with dsDNA) irrespective of the nucleotide sequence of the DNA, and as such, these enzymes belong to the class of non-specific ssDNA binding proteins.

Apart from DNA helicases, RNA helicases as well as enzymes that unwind RNA/DNA hybrids have also been isolated from various species. These are considered to play important roles in gene transcription, in mRNA translation or in RNA splicing (20,21).

I.2. Detection of a Helicase

Under *in vivo* conditions within the cell, a DNA helicase is believed to interact with a topologically complex DNA molecule and is expected to unwind considerably long stretches of the molecule depending on the particular DNA metabolic process in which the particular helicase plays a role. This extensive DNA unwinding is often achieved not only as a result of the intrinsic processivity of the unwinding action of the helicase, but also due to the stimulatory effects deriving from the multiple interactions between the helicase and several other cellular factors. Some of these factors may also stimulate the activity of the helicase, without necessarily interacting with it, by acting on the DNA molecule.

However, the prevailing conditions for enzyme activity assays *in vitro* are hardly similar to those obtainable in the cell and consequently DNA helicases, under these assay conditions, may be expected to function only to a limited degree of their maximal *in vivo* potential. Hence the basic biochemical helicase reaction measures the displacement of a relatively short end-labelled oligonucleotide, in the presence of ATP and Mg^{2+} , from a partial duplex DNA molecule. From the foregoing consideration of the possible effects of protein-protein interactions on the DNA unwinding activity of a helicase in the cell, two extreme situations may be envisaged: a highly processive enzyme which, *in vivo*, unwinds dsDNA without interacting with other cellular proteins or factors would accomplish the same extent of DNA unwinding *in vitro* as *in vivo*. Conversely a helicase that unwinds duplex DNA only when stimulated by other proteins would hardly displace any stretch of oligonucleotide in *in vitro* assays. The exact reality of DNA unwinding by helicases *in vitro*, for the numerous enzymes so far described in the literature, lies between these two extreme examples.

The DNA substrate commonly used to detect helicase activity, *in vitro*, consists of a large single stranded circular DNA molecule (generally the closed form of M13, a bacteriophage of *E.coli*) to which a short end-labelled oligonucleotide is annealed to form a partial duplex. A schematic representation of such substrate used in an *in vitro* helicase assay is shown below in figure 1.2.1.

A preparation of protein containing the enzyme under study is incubated with the DNA helicase substrate in opportune buffer conditions in the presence of ATP and Mg^{2+} at 37°C. Interaction of the supposed helicase with this substrate, in these assay conditions, results in the unwinding of some molecules of this partial duplex substrate and consequent displacement of the oligonucleotide. Subsequent electrophoresis of the reaction mixture on

non-denaturing polyacrylamide gel reveals the displaced oligonucleotide as a radioactive band which migrates faster than the native DNA substrate.

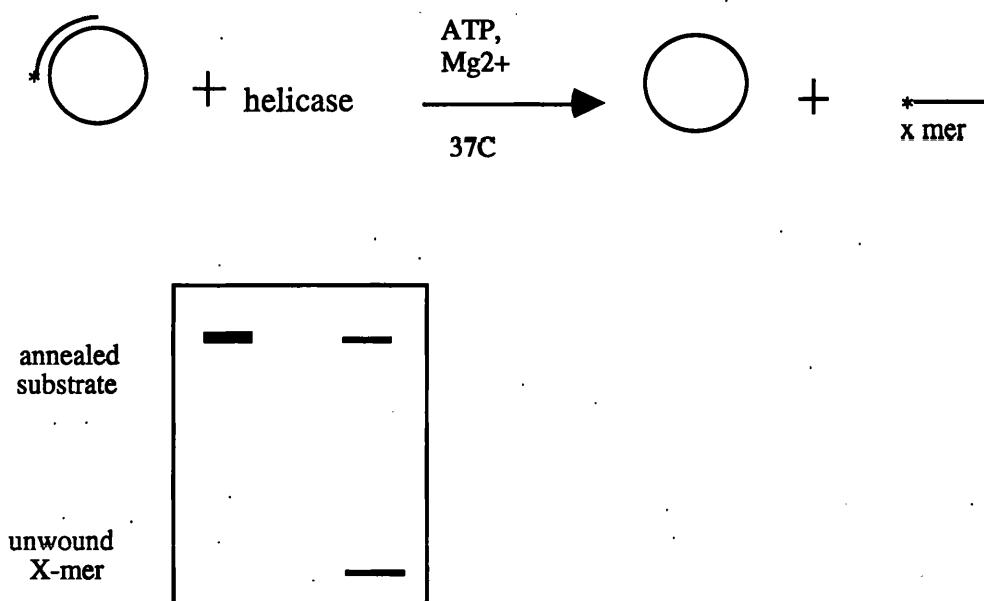


Figure 1.2.1. Helicase Assay: An aliquot of a sample preparation of the protein is mixed with the DNA helicase substrate and incubated under the specified assay conditions. In the presence of a DNA unwinding enzyme, the oligonucleotide is displaced from the substrate of partial duplex DNA, in an ATP-dependent manner, as shown in the figure.

1.3. Polarity of Translocation of Helicases

Helicases generally translocate unidirectionally along the DNA single strand to which they bind while unwinding the duplex region of the DNA. The direction of this movement, with respect to the sugar-phosphate backbone of the DNA, is known as the polarity of translocation of the enzyme. We can therefore distinguish between enzymes possessing a 3' to 5' polarity and those with a 5' to 3' polarity. The DNA substrate generally used to determine enzyme polarity, shown below in figure 1.3.1, consists of a linear DNA molecule carrying an end-labelled oligonucleotide at each of its extremes. These oligonucleotides may be equal in length to each other or may have different lengths from each other, and each may carry a hanging tail for those enzymes that require such a substrate for their activity *in vitro*. The length of these hanging tails, however, should be such as not to allow enzyme binding since this eventuality would cause ambiguity in the determination of enzyme polarity. In the DNA unwinding reaction depicted here, displacement of the fragments X and X' denotes an enzyme that possesses a 3' to 5' polarity, while displacement

of the fragments Y and Y' reveals an enzyme that exhibits a 5' to 3' polarity. However, at least two DNA helicases, namely, the *E.coli* RecBCD complex and one of the enzymes described in this thesis have been shown to exhibit a double polarity of translocation on the DNA. That means that tested under these assay condition, these two enzymes would each displace both fragments X and Y or X' and Y'.



Figure 1.3.1. Polarity of unwinding of helicases: a helicase reacts with the direction-specific substrate. Displacement of the fragments X and Y denotes a helicase moving in the 3' to 5' direction, while the unwinding of the fragments X' and Y' indicates a 5' to 3' translocating helicase.

1.4. Classification of Helicases

Different attempts have been made, by different investigators, to classify DNA helicases. These attempts at classification have largely been based a) on the presence of specific sequence motifs in the amino acid sequences of these enzymes, b) on the macroscopic reaction mechanism exhibited by the enzymes, or c) on their direction of movement on the DNA molecule. However, since each of these methods of classification considers only one feature of the enzymes, it is obvious that none is really satisfactory for ordering in a significant way all the helicases so far isolated from various species and characterized. Some of these classifications are described here:

a. Classification based on amino acid sequence: this method takes into consideration the presence of seven blocks of amino acid sequences (motifs I-VI) in the primary structure of various enzymes and classifies helicases into three vast principal superfamilies (SF1, SF2 and SF3), and two smaller families (F4 and F5) (22). Each of the superfamilies is further subdivided into several smaller families to reflect more detailed sequence similarities among members. The largest of these super families contains the DEAD/H families, the RecQ family, the Snf2-like family, the ERCC3-like family, thus showing little consideration for functional homology among members. While it can be admitted that this method of classification facilitates the allocation of a helicase whose amino acid sequence is known, it must be said that it creates room for possible false positives. These are represented by proteins with considerable amino acid sequence homology with known helicases, by virtue of the presence of the amino acid motifs in their sequence, but which fail to exhibit DNA unwinding activity *in vitro*. These enzymes are often defined as putative helicases, in a somewhat arbitrary way, since no functional or molecular justification has been outlined so

far for the possible role of those sequence motifs in the unwinding reaction. This classification also fails to accommodate and classify those enzymes with proven helicase activities but whose amino acid sequences have, as yet, not been determined. Finally it leaves out documented DNA unwinding enzymes whose amino acid sequences do not carry the characteristic motifs I-VI on which the classification is based.

b. Classification based on enzyme function: this method of classification would recognise four classes of helicases according to the DNA process in which the enzyme plays a part *in vivo*. It can distinguish between enzymes that function in replication such as the SV40 large T antigen and the yeast DNA2 helicase; enzymes active in transcription such as the helicase components of the human TFIIH; enzymes that are involved in recombination such as the *E.coli* RecQ and the RecBCD complex, and finally helicases that function in DNA damage repair such as the human XPB and XPD helicases. Although this system of classification argues for a better method of comparison between helicases from different organisms, it creates some ambiguity since some enzymes may participate in more than one cellular process and would, therefore, belong to more than one class of helicases. Such is the case of the helicase components of the human transcription factor IIIH (TFIIH), a multi-protein complex known to be involved both in transcription and in the repair of damaged DNA (23). But more importantly, this system leaves out a large number of helicases, especially eukaryotic enzymes, for whom *in vivo* functions are yet to be determined.

c. Classification based on mechanism of reaction: helicases may also be classified according to their macroscopic reaction mechanism while unwinding DNA duplexes. We can distinguish among three such classes: enzymes with a catalytic mechanism, stoichiometric enzymes, and enzymes that show a limited reaction mechanism (4). A catalytic enzyme unwinds the same fraction of each substrate at a given enzyme concentration independent of the length of the duplex region. For a stoichiometric helicase, the fraction of substrate unwound decreases progressively as the length of the duplex region increases. In the case of enzymes with a limited mechanism of reaction, the fraction of substrate unwound decreases dramatically as the duplex region increases. According to this method of classification, the majority of isolated helicases belong to this last class of enzymes at least in *in vitro* helicase assays.

d. Classification according to enzyme polarity: in the reaction of dsDNA unwinding, helicases are believed to translocate uni-directionally along the DNA strand to which they bind. These enzymes may therefore be grouped into two classes: those possessing a 3' to 5' polarity and those that exhibit a 5' to 3' polarity. Although this method of classification may seem to embrace all known helicases, at least two enzymes, the *E.coli* RecBCD complex and one of the enzymes described in this thesis have been shown to exhibit an double polarity while unwinding the double strands of the DNA molecule (24).

1.5. Oligomeric Nature of Helicases

Helicases may be isolated in different conformational states but several studies have reported that these enzymes usually assemble into complexes, generally dimers or hexamers, at their site of action *in vivo*. This oligomerization is believed to be the effective functional conformation state of these enzymes induced in the presence of the DNA substrate as is exemplified by the dimerization of the *E.coli* Rep helicase (25); or also in the presence of NTP co-factors and divalent cations, since this has also been observed for some helicases *in vitro*, as in the case of the SV40 T antigen (26). The immediate consequence of such oligomeric structures is that helicases acquire multiple DNA binding sites. These binding sites appear necessary for the simultaneous binding of different regions of the DNA by the helicase (single strand, double strand and single strand/double strand junction) during the unwinding reaction according to the proposed mechanisms of helicase-catalyzed DNA unwinding (27). Table 1.5.1 lists a few helicases with established *in vivo* functions and known to assemble into multi-subunit complexes at the site of DNA unwinding.

TABLE 1.5.1 Oligomeric Nature of Helicases

Helicase	Assembly state	Polarity of unwinding	Function
<i>E.coli</i> DnaB	hexamer	5' to 3'	replication
SV40 large T antigen	hexamer	3' to 5'	replication
T7 phage gene 4 protein	hexamer	5' to 3'	replication
T4 phage gene 41 protein	hexamer	5' to 3'	replication
<i>E.coli</i> Ruv B	hexamer	5' to 3'	recombination
<i>E.coli</i> Rho protein	hexamer	5' to 3'	transcription
<i>E.coli</i> RecBCD complex	heterotrimer (or hexamer)	double	recombination
<i>E.coli</i> Helicase II	dimer (or larger oligomer)	3' to 5'	DNA repair
<i>E.coli</i> Uvr AB	hetero-trimer (A ₂ B)	5' to 3'	DNA repair
Herpes simplex (HSV-1) UL9	dimer	3' to 5'	replication

1.6. Mechanism of DNA Unwinding by Helicases

Helicases bind to different portions of the DNA molecule (single strand regions, and single strand/double strand junctions) and melt the inter-strand hydrogen bonds as they move along the DNA molecule. These enzymes must, therefore, contain the requisite DNA binding domains within their structures. The energy necessary for the movement on the DNA and for the dissociation of these hydrogen bonds is supplied by the hydrolysis of nucleoside triphosphates co-factors (NTPs), implying that helicases also bind to NTPs. The binding and hydrolysis of NTPs induce conformational changes in the helicase molecule and consequently modulate its affinity for the DNA (27). Although this phenomenon has been proposed as the macroscopic mechanism of enzyme movement on the DNA molecule, the intimate aspects of DNA unwinding and helicase translocation still remain unclear. It is, however, believed that although the detailed mechanisms of action may differ for different helicases, some general features of the process of DNA unwinding may be common to all.

Two principal models have been proposed to explain the mechanism by which DNA helicases couple NTP binding and hydrolysis to translocation on the DNA (27), namely, the "rolling" model and the "creeping" (or inch-worm) model. Both models require the presence of at least two DNA binding domains on every functional helicase entity to guarantee the simultaneous binding of the helicase to different regions of the DNA substrate.

a. The "rolling" model for helicase-catalyzed DNA unwinding is represented schematically in figure 1.6.1 and depicts the mode of DNA unwinding and translocation by the homo-dimeric *E.coli* Rep helicase.

This model requires the presence of multiple identical DNA binding sites in the functional helicase entity and, although such binding sites can be located on the same enzyme molecule, the "rolling" model of DNA unwinding requires the formation multimeric enzyme structures according to the proposed oligomeric nature of helicases. This model further implies that each subunit of the helicase oligomer should be able to bind both single stranded (ss) and double stranded (ds) DNA, and that the functional helicase binds to both regions of the DNA simultaneously during, at least, one intermediate phase of the unwinding reaction. The "rolling" model of DNA unwinding was proposed for the Rep helicase on the observation that binding to DNA induces the enzyme to dimerize (25) and that nucleotides allosterically influence its affinities for single stranded and double stranded DNA. In the DNA unwinding reaction considered here, the helicase action begins with both subunits of Rep binding to the 3' single stranded portion of the DNA (intermediate I). Binding of ATP causes a decrease in the affinity of one of the subunits of Rep for ss DNA, and at the same time, its affinity of ds DNA increases. This leads to the intermediate (III) in which one subunit of the enzyme binds to the double stranded portion of the DNA ahead of the fork of unwinding while the other subunit stays bound to the single stranded DNA region.

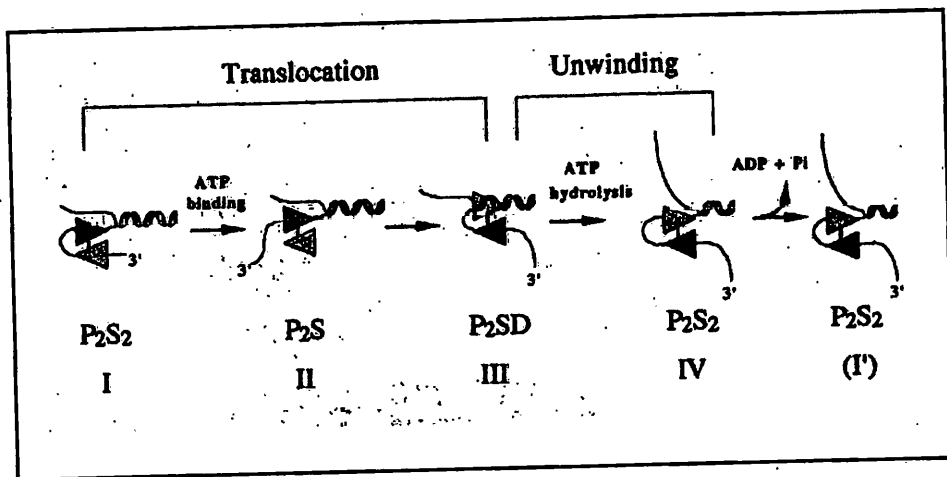


Figure 1.6.1: "Rolling" mechanism for Rep-catalyzed DNA unwinding. The two Rep monomers are distinguished (black and grey) to indicate how the positioning of each monomer changes during unwinding

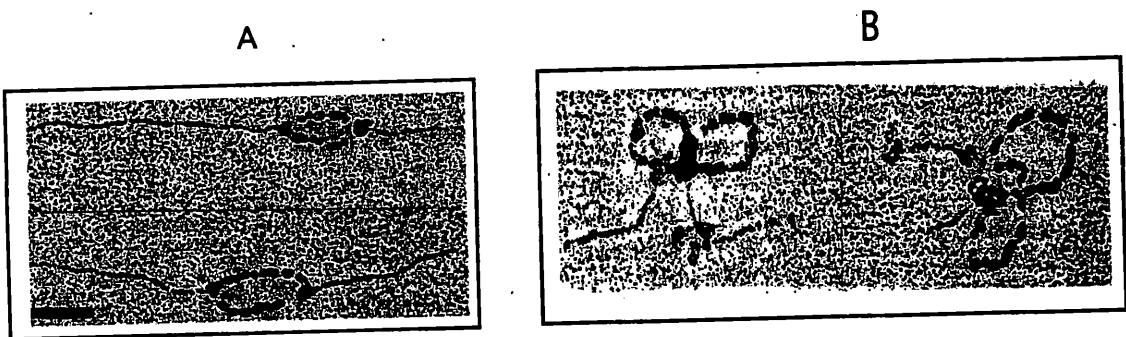


Figure 1.6.2 : Electron micrographs of unwinding intermediates started from the SV40 origin of DNA replication. (A) Unwinding bubbles, fully expanded, which arose by bidirectional unwinding started from the origin. The individual T-antigen complex is bound at the branch point of both forks; (B) "Rabbit-ears" containing structures. The bi-lobed complex at the center of the unwinding intermediates connecting both forks thereby causing the extrusion of two ssDNA loops, is T-antigen.

Subsequent hydrolysis of ATP induces conformational changes in the helicase and this in turn destabilizes several base pairs in the DNA substrate (intermediate IV). The final release of ADP and inorganic phosphate re-establishes the initial situation of DNA binding and thus completes the cycle of DNA unwinding. Therefore, in this model of unwinding, translocation of the Rep homo-dimer on the DNA is coupled to ATP binding whereas actual DNA unwinding is coupled to hydrolysis of the NTP co-factor. However, the recent resolution of the crystal structures of PcrA and HCV NS3 has shown that the active form of these enzymes are monomers, in contrast to the oligomerization of helicases required by the "rolling" model for DNA unwinding (28,29).

b. The "creeping" (or inch-worm) model for DNA unwinding differs from the "rolling" model by proposing the presence of non-identical DNA binding domains in the functional helicase entity, each of which binds either the double stranded or the single stranded DNA region. These enzymes may therefore function as monomers since the requisite DNA binding sites can exist in the same protein molecule.

The "creeping" model for DNA unwinding, therefore recognizes a leading domain in the helicase (H) which remains permanently bound to the double stranded portion of the DNA but which can also bind to single stranded DNA, and a tail portion (T) which binds only to ssDNA. ATP binding and hydrolysis alter the affinity of the leading portion (H) for dsDNA and also causes the destabilisation of this portion of the DNA. Release of ADP and inorganic phosphate causes an increase in the affinity of the leading portion of the enzyme (H) for dsDNA. The helicase then creeps onto the proximal double stranded portion of the DNA to be unwound and therefore translocates on the DNA molecule. In this model, DNA unwinding is also connected to binding and hydrolysis of the NTP co-factor while enzyme movement is caused by the release of the products of hydrolysis of NTP.

The "rolling" and "creeping" models for DNA unwinding may account for the mechanism of action of helicases since these enzymes are believed to translocate along the single stranded DNA while unwinding the double stranded region as they move along. An entirely different model has been proposed by Wessel et al (30) for the mechanism of DNA unwinding by hexameric helicases such as the SV40 large T antigen. Although no crystal structures are yet available for these hexameric helicases, it is known that they form a ring-like structure around the DNA double strand and that the DNA transits through this ring-like structure in order to be unwound. The mechanism of DNA unwinding by such enzymes therefore, appears different from both the "rolling" and "creeping" models. In fact, by electron microscopy, these authors observed that in the presence of ATP and Mg^{2+} , the SV40 T antigen assembled into a large dodecamer complex (double hexamer) at the viral origin of replication and took part in the initial denaturation of the origin. Furthermore, they observed that after the activation of the origin, this dodecamer complex bound to the origin did not split into two hexamers that would translocate along and unwind ds DNA, rather dsDNA was threaded through the intact dodecamer complex with extrusion of single

stranded loops, to form structures like “rabbit ears” from both ends of the dodecamer complex. According to this model, a schematic representation of which is shown in figure 1.6.2, the helicase entity does not move but stays fixed at the region of the origin while dsDNA moves through the complex for the unwinding reaction to occur.

A similar model has been proposed also for the unwinding action of *E.coli* DnaB and PriA helicases at the fork of DNA replication. Figure 1.6.3 shows the unwinding action of *E.coli* DnaB and PriA helicases exerted in opposite directions to guarantee the simultaneous syntheses of both the leading and lagging DNA strands. Both helicases remain bound to each other and therefore “pull” the DNA strands rather than translocate on the DNA molecule. Jezwska et al have suggested the passage of the unwound ssDNA through the ring-like structure formed by the hexameric DnaB helicase during DNA unwinding (31).

Thus, although the “rolling” and “creeping” models may appear attractive to explain the mechanism of helicase-catalyzed DNA unwinding, it is clear that other alternative mechanisms may also exist and unless further evidence supports any particular model, there is no reason to adopt any as the sole model for the mechanism of DNA unwinding by helicases.

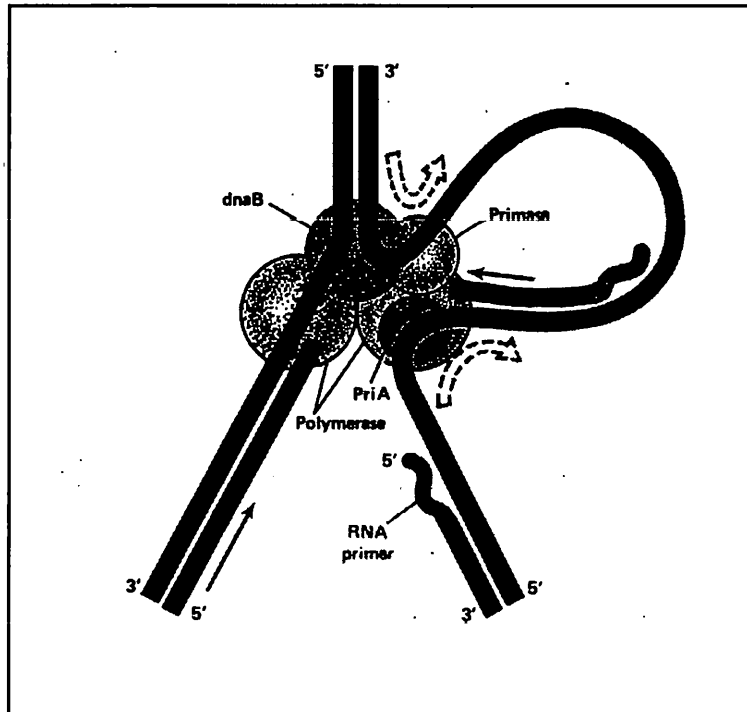


Figure 1.6.3: Model for simultaneous replication of leading and lagging strands by a DNA polymerase associated with a primosome to form a "replisome". Note that the DnaB and Pri A helicases act on the DNA template in opposite directions. In this model of DNA unwinding, the helicases seem to pull the DNA strands rather than translocate on them.

2. IN VIVO FUNCTIONS OF HELICASES

Nearly all the DNA helicases characterized in *E.coli* have defined *in vivo* functions. *E.coli* DnaB and PriA are helicases involved in DNA replication (32,33), and the function of DnaB is regulated by the DnaG gene product (34). Rec Q and Rec BCD complex are involved in recombination, while UvrAB, UvrD are repair helicases (35,36). In the simian virus 40, the viral large tumour antigen is the only viral protein (a helicase) involved in DNA replication (30). *In vivo* functions have also been determined for some helicases of lower eukaryotes, notably the yeast *saccharomyces cerevisiae*. Yeast DNA2 helicase functions in replication (37) while RAD3 and RAD25 are both helicases operating in genetic recombination (38). However, very little is known about the biological functions of the majority of the helicases isolated from higher eukaryotes, especially from human cells. In these organisms, in fact, attribution of *in vivo* functions to helicases has often been inferred from comparison of their *in vitro* biochemical features with those of corresponding enzymes in prokaryotes or in lower eukaryotes for which *in vivo* biological functions have been defined. However, caution should be exercised in interpreting processes in higher eukaryotes with the knowledge of similar processes in prokaryotes since more complex genome organization, in eukaryotes, may have led to greater specificities in enzyme function unparalleled in prokaryotes.

One of the biggest problems in attributing *in vivo* roles to helicases isolated from higher eukaryotes is the lack of adequate *in vitro* assays that portray enzyme functions *in vivo*. This discrepancy in turn derives from the difficulty in setting up *in vitro* conditions that faithfully mimic the *in vivo* ones. Virtually all helicases in the cell operate in concert with numerous other cellular factors as members of multi-protein complexes (39,40), hence the difficulty in assigning roles to isolated enzymes. In fact, eukaryotic helicases with established *in vivo* functions were first characterized as members of multi-protein complexes involved in a certain cellular process and subsequently identified as DNA unwinding enzymes. Examples are the multi-protein transcription factor II H (TFIIH) which contains a helicase (39) and the multi-protein complex of *S. cerevisiae* which is involved both in transcription and DNA repair (40). The nucleotide excision repair machinery contains the helicases XPD and XPB (41,42) which are also involved in transcription; the DNA-PK/Ku complex involved in V(D)J recombination and which also phosphorylates numerous cellular factors contains the human DNA helicase II (HDH II) (43). Helicase activity is associated with some members of the MCM group of proteins (44), a multi-protein complex widely documented to be involved in the initiation of DNA replication (45-46).

The roles played by some helicases in some key cellular processes will be briefly discussed here.

2.1. Helicases and Gene Transcription

Except during mitosis, the only cell cycle phase in which the cellular transcriptional machinery is completely switched off, gene transcription is a continuous process even in quiescent cells. The genes which are preferentially expressed in quiescent cells have been designated "growth arrest specific genes" (GAS) and their expression seems necessary for the maintenance of quiescence (47,48), but these are far from being the only genes transcribed in these conditions. Transcription may be divided into three phases each of which represents a different stage of possible interaction between a helicase and the DNA, namely, transcription activation, elongation of the RNA and transcription termination.

Transcription activation starts with the recognition of, and binding to a specific promoter sequence by the RNA polymerase. A binary complex is formed between the DNA template and the RNA polymerase, and the first ribonucleotides are incorporated.

The elongation of the nascent RNA leads to the formation of a ternary complex between the template DNA, the RNA polymerase and the RNA chain in the transcription bubble, and the simultaneous unwinding of the RNA/DNA hybrid at the 5' terminus of the nascent RNA molecule.

Termination of transcription brings about the dis-assembly of the transcription machinery with consequent release of the newly synthesized RNA chain.

In *E.coli* however, it is not quite clear whether the unwinding events at the early stages of transcription are the consequences of thermal fluctuations in the region of the DNA or caused by the action of DNA helicases. Von Hippel et al (49) have proposed a model of gene transcription in *E.coli* in which template DNA unwinding ahead of the polymerase and rewinding behind it is achieved by a rotation of the axis of the template with respect to the enzyme in the transcription bubble. Likewise by rotation, the newly synthesized RNA is unwound at the 5' end of its hybrid with DNA leading to simultaneous elongation at the 3' end. This model is illustrated schematically in figure 2.1.1.

a. in *E.coli*: the most characterized bacterial transcription helicase is the rho protein (50,51) which is involved in the termination of RNA polymerase-catalyzed transcription. Unlike many helicases, the rho protein seems to recognize and bind to some discreetly specific RNA sequences located 5' to rho-dependent termination sites within the transcription bubble. Although lacking extensive sequence homologies, these binding sites (also known as rho loading sites) are generally devoid of RNA secondary structures and are rich in cytosine residues (52). Rho protein is an RNA/DNA helicase with a 5'-3' polarity. It requires a single-stranded RNA tail to bind and seems incapable of unwinding RNA duplexes since a short stretch of RNA duplex can block its helicase activity (52). From a structural point of view, the

rho protein is a hexamer of identical subunits organized as a trimer of asymmetric dimers with an overall three-dimensional symmetry. Thus each dimer has one strong and one weak ATP binding site and one weak and one strong RNA binding site. Each subunit of the hexamer has three functional domains: the RNA recognition/binding domain is located in the N-terminal region; the ATP binding site is in the middle portion of the protein while the subunits interact with one another through their respective C-terminal portions (53). The binding of rho protein to ATP and to RNA induces conformational changes in the enzyme, and since ATP hydrolysis modifies the affinity of a given rho dimer for RNA, the ATPase activity of the enzyme has been proposed to generate the energy for rho translocation on the nucleic acid. Although details of this process are unclear, the integrity of the ATP binding site of the protein remains fundamental to its helicase activity. In fact a single amino acid mutation in this region modifies the RNA binding properties of the protein and severely impairs the release of the newly synthesized RNA from the RNA/DNA hybrid at a rho-dependent transcription termination site (54).

Geiselman et al have proposed a physical model for the functions of the rho protein (53) according to which, the translocation of the enzyme along ssRNA in the 5' to 3' direction is guaranteed by a switch in the conformational states of the rho monomers for RNA, from the state of high affinity to low affinity. This is brought about by ATP binding to RNA-bound rho: the rho monomer which is bound to the 5' end of the RNA switches from the state of high affinity for RNA to that of low affinity and, therefore, releases this segment of the nascent RNA. Subsequent binding of this free rho dimer to the 3' segment of the RNA re-establishes the initial symmetry in the hexameric molecule and causes the enzyme to advance by a step-size equal to the size of the enzyme dimer on the RNA molecule. Repetitive dimer-size translocations brings the enzyme into contact with the ternary transcription complex (polymerase/DNA template/RNA chain) paused at a rho-dependent termination site. Transient destabilization of this complex which is partly due to thermal fluctuations and also to the proximity of the enzyme leads to exposure of segments of the nascent RNA. By occupying these freed segments, the enzyme accomplishes its RNA/DNA helicase function which ultimately leads to the release of the RNA transcript from the DNA template.

b. in eukaryotes: transcription in eukaryotes presents the same stages of activation, elongation and termination described above for prokaryotes, but the understanding of the mechanisms involved in each step is far less complete than in bacteria. A few eukaryotic helicases and putative helicases involved in transcription have been described (55,56). In the yeast *S. cerevisiae*, some of these enzymes have been identified through analyses of the effects of mutations in their genes on transcription (57,58); therefore, knowledge of the roles they play in the process of transcription has not gone much beyond their mere identification. Sopta et al have described the DNA helicase activity of the human RAP 30/74 protein, a general transcription initiation factor that binds to RNA polymerase II (59). Whereas various reports fail to associate helicase activity with transcription factors isolated from yeast despite

hydrolysis of NTP co-factors by these enzymes (60,61), these authors have documented the energy-driven melting of the dsDNA template by RAP 30/74 to generate the single stranded template necessary to form the transcription initiation complex (59). This enzyme, however, does not possess the RNA/DNA unwinding activity required to liberate a newly synthesized RNA chain from its hybrid with DNA at a transcription termination site.

The yeast transcription factors SNF2/SWI2, STH1 and MOT1 contain the seven amino acid motifs characteristic of helicases. SNF2 shows DNA-dependent ATPase activity but does not unwind dsDNA *in vitro* (60). Laurent et al have suggested that possible DNA unwinding by SNF2 in transcription might require the presence of other SNF/SWI proteins such as SNF5 and SNF6 (62).

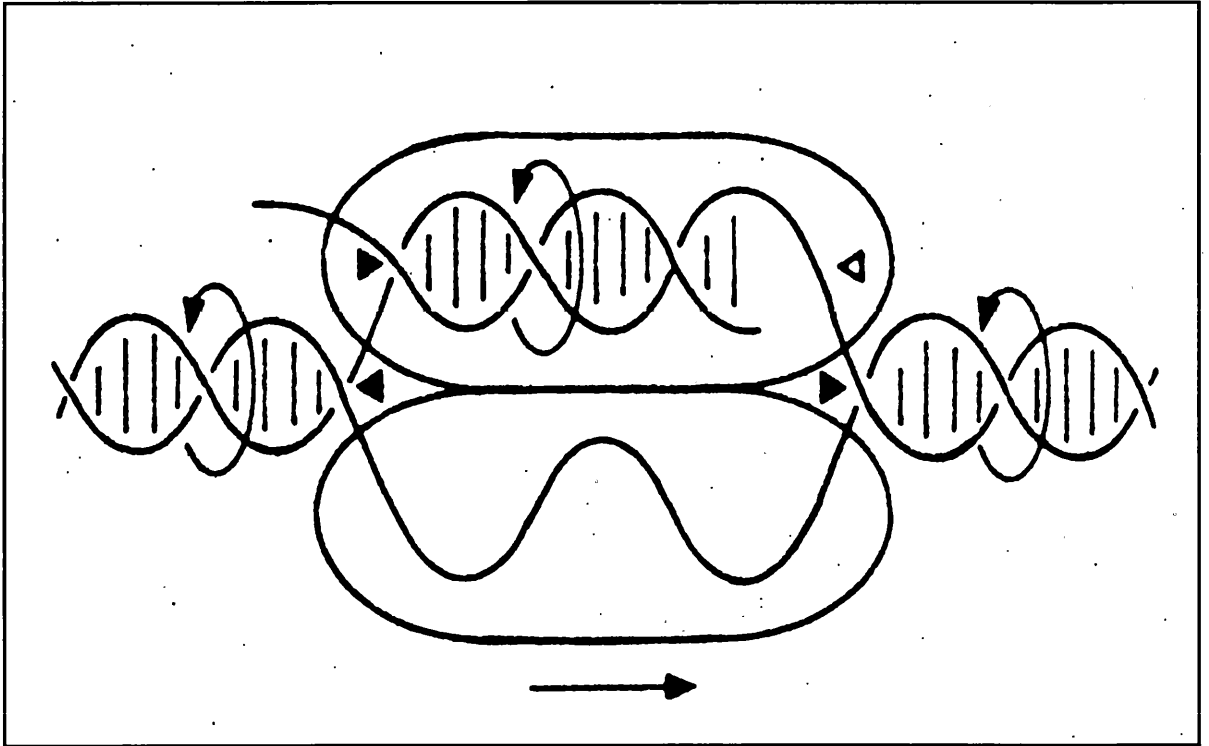


Figure 2.1.1. DNA Unwinding in transcription in E.coli: Template DNA is unwound through rotation of its axis to provide the transcribed single strand. Likewise by rotation the DNA is rewound behind the transcription machinery to free the 5' terminus of the nascent RNA molecule.

2.2. Helicases and DNA Damage Repair

The integrity of the genetic material in DNA is continually threatened by a variety of environmental agents such as UV irradiation, various chemicals and also through faulty metabolism of the DNA all of which cause damage to the DNA molecule. If these lesions are not removed they can disturb fundamental cellular processes such as transcription or replication since replication of damaged DNA would give rise to mutations. Cells have therefore developed various mechanisms to repair damaged DNA, the most important and most studied of which is the nucleotide excision repair mechanism (NER). NER is capable of removing many different DNA lesions among which are UV (sunlight)-induced cyclobutane Pyrimidine dimers (CPD), bulky chemical adducts and pyrimidine (6-4) pyrimidone photo-products.

Nucleotide excision repair mechanism can be schematically divided into five steps:

- recognition of damage on the DNA
- incision of the damaged strand on both sides of the lesion
- excision of the damaged strand
- DNA repair synthesis to fill the single-stranded gap
- ligation of the newly synthesized strand to the parental DNA

Helicases involved in DNA repair are, therefore, members of multi-enzyme complexes even though not all the components of such complexes are needed to initiate the repair process. Some are recruited as the need arises for their functions while others dissociate on completion of their specific roles.

a. In *E.coli*: at least six proteins are involved in the nucleotide excision repair process in *E.coli*, namely UvrA, -B, -C, -D, DNA polymerase I and DNA ligase. These bacterial proteins have been purified and the genes encoding them have also been isolated (63). A simplified scheme of this repair process is shown in figure 2.2.1.

In *E.coli*, the nucleotide excision repair mechanism starts when UvrA dimerizes, in the presence of ATP and recruits one molecule of UvrB to form the complex UvrA₂B. This complex possesses a mild 5' to 3' translocation ability by virtue of which it can translocate on DNA in search of a damaged segment (64,65). The UvrA₂B complex is believed to recognize a structural distortion on the DNA helix rather than the damaged base/s, and this explains the broad spectrum of DNA damage removable through NER. Upon encountering damage, a stable pre-incision complex is formed with the DNA at the site of the lesion and UvrA probably dissociates from the complex. Formation of this complex between UvrB and the DNA brings UvrC to bind and stimulates the dissociation of UvrA. Thereafter UvrB nicks the damaged strand at the 5th phospho-diester bond 3' to the lesion whereas UvrC incises the DNA strand at the 8th phospho-diester bond 5' to the damaged base (66). The resulting oligonucleotide as well as the UvrC protein are displaced by the 3' to 5' translocating UvrD

helicase while DNA polymerase I synthesizes a new strand and causes the removal of UvrB. The repair reaction is completed when the newly synthesized strand is ligated to the parental DNA by DNA ligase.

b. In Eukaryotes: nucleotide excision repair is an evolutionarily conserved process and is the most important mechanism for the repair of damaged DNA also in eukaryotes. However, knowledge of the detailed processes involved in eukaryotic NER is much less complete than in prokaryotes. In humans, NER deficiency gives rise to the insurgence of at least two clinical syndromes: Xeroderma pigmentosum (XP) and Cockayne's syndrome (CS) (15). XP patients are extremely photosensitive, they exhibit pigmentation abnormalities in sun-exposed areas of the skin and have a high risk of developing skin tumours. XP patients also suffer from progressive neurological abnormalities (intellectual deterioration and loss of speech) which are believed to be due to neurodegeneration. Patients with Cockayne's syndrome are also photosensitive but have less risk of developing skin cancers (15). However, these patients also manifest neurological disorders similar to XP patients. Studies conducted on several cases of naturally existing human NER deficiencies have led to the identification of seven complementation groups for xeroderma pigmentosum (XP-A to -G) and two for Cockayne's syndrome (CS-A and CS-B) denoting the impaired steps of this DNA repair pathway (67). Genomic DNA transfections into (laboratory-induced) UV-sensitive CHO cell lines have led to the isolation of the human genes that correct the relative rodent repair deficiencies and which have been subsequently termed excision repair cross-complementing rodent repair deficiency genes (ERCC) (68-71).

At least ten genes involved in nucleotide excision repair have been isolated in the yeast *Saccharomyces cerevisiae* (72) and some of the products of these genes have conserved both sequence and functional similarities with their mammalian homologues. Knowledge on their functions will therefore be described alongside their mammalian counterparts. An example is the yeast RAD25 protein which possesses 55% identical and 72% conserved amino acid residues with the human nucleotide excision repair protein XPB. Since a mutation in the C-terminal region of RAD25 conferred uv sensitivity, this protein was suggested to function in NER in yeast, in analogy to the human repair protein XPB (73). Although as I mentioned earlier, details of the molecular mechanisms of the eukaryotic NER are much less understood than in *E.coli*, the overall reaction stages are believed to be the same in all species. Equally conserved are the functions of the various proteins involved in DNA repair in both systems, not surprisingly some of the eukaryotic NER gene products have been shown to possess DNA unwinding activities (74,75). The available data on the identification, characterization as well as the biochemical analysis of NER factors in eukaryotes have been compiled into a model for the mechanism of nucleotide excision repair of damaged DNA in the same system.

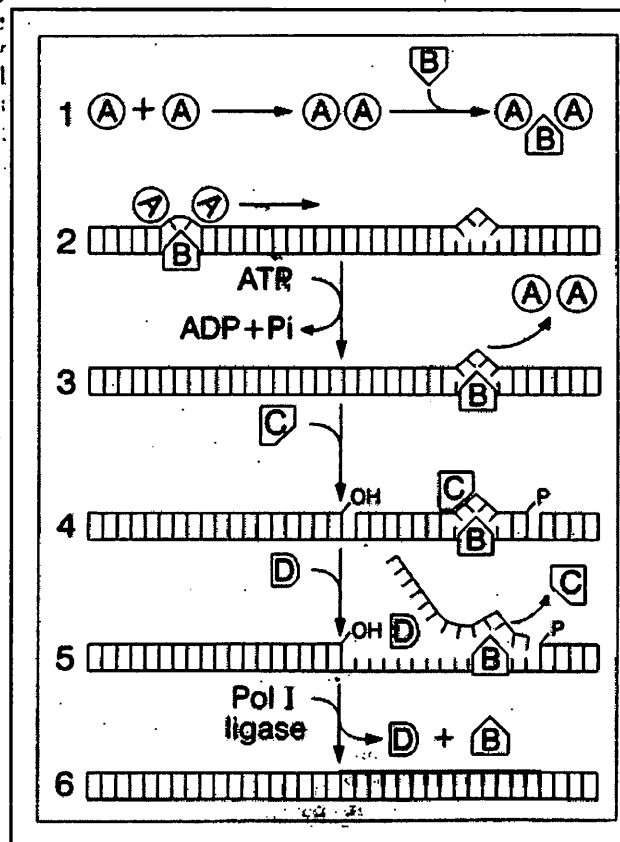


Figure 2.2.1. Model for the reaction mechanism of *E. coli* nucleotide excision repair: 1. formation of UvrAB heterodimer, 2. UvrAB scanning of DNA, 3. UvrB capture and release of UvrA, 4. UvrC binding and incision of oligonucleotide by UvrD, 6. DNA synthesis and ligation

1. recognition of DNA damage: this is the first step in NER, and two human proteins, XPA and XPE have been proposed as candidate factors, both are DNA binding proteins with high affinity for UV-damaged DNA. However, XP-E patients exhibit a mild form of xeroderma pigmentosum and their *in vivo* repair processes are approximately 50% of the observed repair in normal individuals (76). This finding makes XPE a less appropriate candidate (compared to XPA) for the basal detection of DNA damage. The *S. cerevisiae* sequence and functional homologue of XPA is RAD 14 which contains 27% identical and 54% similar amino acid residues to the human protein (77). RAD 14 also binds damaged DNA preferentially in a manner similar to XPA.

2. melting of Duplex DNA: in analogy to the functions of UvrA₂B and UvrD in *E. coli* NER, helicases are expected to play similar roles in the eukaryotic system. XPD/ERCC2 and XPB/ERCC3 are evolutionarily conserved proteins, and their amino acid sequences contain the seven conserved motifs characteristic of many helicases (69,70). These enzymes have, therefore, been proposed as DNA helicases involved in the eukaryotic nucleotide excision repair machinery. The *S. cerevisiae* homologues of these two proteins are Rad3 and Rad25, and interestingly, purified Rad3 and Rad25 proteins both possess ATP-driven DNA unwinding activity (78) with a 5' to 3' and a 3' to 5' polarity respectively and similar unwinding activity has also been reported for their human homologues, XPD, XPB. Furthermore, the ATPase and helicase activities of Rad3 are inhibited after UV irradiation of the substrate and the protein seems to be sequestered to the site of DNA lesion (79). XPD/ERCC2 and Rad3 have been proposed as the eukaryotic proteins with analogous functions to the *E. coli* repair helicase UvrA₂B, while Rad25 and XPB/ERCC3, both possessing a 3'-5' helicase activity, have been proposed as analogues of the UvrD helicase.

3. excision of Damaged DNA, the 3' and 5' endonucleases: after the DNA unwinding reaction by the helicase, release of the damaged DNA segment requires the site-specific incision of two endonucleases on both sides of the damage. In mammalian NER, the 3' incision is accomplished by XPG/ERCC5 while XPF/ERCC4 incises the damaged DNA strand at the 5' side of the lesion. The *S. cerevisiae* functional analogues of the human proteins are Rad2 and the Rad1/Rad10 complex respectively (80,81).

4. filling the gap and ligation: these are the final steps of the nucleotide excision repair process and are performed by the common DNA replication factors. PCNA actively recruits polymerase ϵ , the most suitable enzyme to synthesize short DNA fragments. Sealing of the newly synthesized fragment to the parental DNA by a DNA ligase completes the repair of damaged DNA by the nucleotide excision repair machinery.

Since the repair of damaged DNA through the nucleotide excision repair mechanism is a very exhausting task, all cells have developed a sub-pathway of NER for the preferential

repair of actively transcribing genes especially when a transcribing gene is blocked at the site of a DNA lesion. This is called "transcription coupled repair" and a good example of this is the release of the RNA polymerase complex stalled at a DNA lesion. Not surprisingly the mammalian basic transcription factor TFIIF/BTF, a multi-protein complex, is also involved in NER. Transcription-coupled repair (TCR) of active genes is considerably faster in all cells than normal NER (82,83). The DNA helicase components of this complex exhibit the 5' to 3' and 3' to 5' polarities reminiscent of XPD and XPB respectively.

2.3. Helicases and Recombination

Recombination is a naturally occurring, enzyme-catalyzed, process by which cells exchange genetic material between homologous DNA molecules. This process, therefore, presupposes the search for similarity between two DNA molecules and the identification of sequence homology before the DNA strands are mutually exchanged. The complex sequence of events that make up the process of recombination may be divided into the following four stages: a) initiation; b) formation of a Holliday junction; c) extension of the hetero-duplex region; d) resolution of the Holliday junction.

The first step in recombination generally involves the introduction of a break (actually a physical damage) to one of the recombining DNA molecules. Therefore, homologous recombination is not only a means of genetic exchange between DNA molecules but also an important cellular process for the repair of damaged DNA. In fact, the site-specific recombination process, the V(D)J recombination, which is a process of antibody gene rearrangement that takes place in the immune system begins with the introduction of a double-strand break between a recombination signal sequence and the adjacent coding sequence, and its overall mechanism evokes the cellular process for the repair of DNA double-strand breaks caused by ionizing irradiation.

a. In *E.coli*: recombination is a complex process requiring the participation of many enzymes and accessory factors. The principal protein involved in recombination in *E.coli* is the RecA protein. This protein forms a nucleoprotein filament with single stranded DNA in the presence of SSB, and thereafter is capable of invading a contiguous dsDNA molecule in search of a homologous DNA sequence. On finding this homology, the RecA nucleoprotein filament is capable of aligning the ssDNA to the homologous sequence to form a portion of hetero-duplex DNA and is capable of inducing DNA strand-exchange between the recombining molecules. However, the initial unwinding of the DNA duplex to generate the suitable single stranded DNA substrate for the RecA protein is operated by the RecBCD complex in the RecBCD pathway of homologous recombination. (84). In fact, this enzyme has been shown to preferentially catalyze the unwinding of blunt-end DNA duplexes and to

be inhibited by stretches of ssDNA regions of up to 25 bases. The RecBCD complex is a three-subunit helicase which also possesses a nucleolytic activity that nicks the exposed single stranded DNA chains asymmetrically (the 3' strand is nicked more frequently than the 5' strand) as the enzyme unwinds the DNA. This nucleolytic activity of RecBCD is, however, down-regulated by a specific DNA sequence called χ (chi, 5'-GCTGGTGG-3') (85). Upon encountering this sequence in the correct orientation, the RecBCD enzyme pauses and nicks the DNA at about six bases to the 3' side of χ . Furthermore, on resumption of DNA unwinding, by the helicase, after the encounter with χ no more broken segments of DNA are observed, rather a stretch of ssDNA is observed to the 5' side of χ and the RecBCD enzyme does not recognize other χ sequences on the same molecule. It has been proposed that the attenuation of the nucleolytic activity of this enzyme, upon interaction with the χ sequence, is related to the displacement of the RecD subunit (86). In fact, this enzyme does not regain its nuclease activity even after it has dissociated from the molecule containing χ and has initiated the unwinding of another DNA molecule. The single-stranded DNA generated by the unwinding activity of the RecBCD constitutes an appropriate binding substrate for the RecA protein to form the nucleo-protein filament. This is shown schematically in figure 2.3.1

As I mentioned earlier RecA protein is the central protein functioning in the process of recombination in *E.coli* and its active form is believed to be the helical filament formed by the binding of this protein to DNA in the presence of ATP and single-stranded DNA binding protein. The RecA-DNA complex invades a contiguous double stranded DNA molecule in search of a homologous sequence. Details of this homology search are not very clear but it is believed that the first steps must involve random non-homologous contacts. Base pairing occurs, a hetero-duplex region of DNA is formed and DNA strand-exchange is induced by RecA when a sufficiently long homologous DNA sequence is found in the recipient molecule. Subsequently, the RecA protein dissociates from the DNA upon hydrolysis of ATP (87). Thus the hydrolytic cycle of ATP modulates the affinity of RecA for DNA: binding of ATP induces the functional state of RecA which has a high affinity for DNA while ATP hydrolysis induces the non-functional state of the enzyme characterized by a low affinity for DNA which causes the enzyme to dissociate. The homologous base pairing and DNA strand exchange between the invading and the recipient DNA molecules leads to the formation of the recombination intermediate called the Holliday junction (a D-loop is formed when only one DNA strand crosses over to the other molecule). The accurate resolution of this structure into mature recombinant molecules is mediated by Holliday junction-specific enzymes (88). In the *E.coli*, these enzymes are RuvA, RuvB, RuvC and RecG. A schematic representation of the various stages of the action of these enzymes on the Holliday junction is shown in figure 2.3.2.

The first step of this process is the extension of the region of recombination between the two molecules. This process, known also as branch migration, is catalyzed both by RecG and by the RuvAB complex, enzymes shown to bind specifically to the Holliday junction.

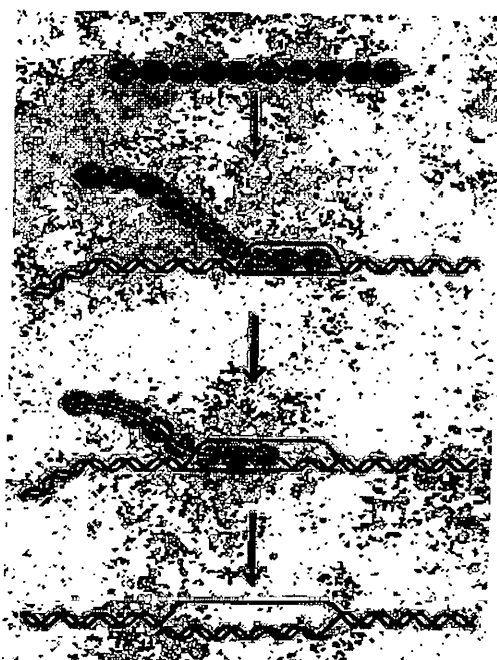
However, whereas RecG is an ATPase capable of driving branch migration by itself alone, RuvAB functions as a complex: the RuvA subunit targets the complex to the junction while the RuvB subunit provides the driving force necessary for branch migration. The RuvAB-Holliday-junction complex consists of two hexameric rings of RuvB surrounding a core of RuvA tetramer (89). Both RecG and RuvAB are helicases (90,91), and both can catalyze branch migration through recombination intermediates coated with RecA protein and actively undergoing DNA strand exchange.

The final step in recombination, the resolution of the Holliday junction, is mediated by the endonucleolytic activity of RuvC. This Holliday-junction-specific enzyme functions as a dimer and cleaves the junction by introducing nicks on the two homologous strands with identical polarity at preferential sites 3' to thymidines (88). Re-ligation of these DNA strands with different partner strands gives rise to mature recombinant molecules.

Other genetic recombination pathways such as the RecE and RecF pathways may be functional alternatives to the RecBCD pathway, but details of their mechanism/s of action, probably revolving around the central function of the RecA protein, are not very clear. These proteins may also be involved in other cellular processes besides recombination (92,93). Likewise other enzymes, notably the RecQ helicase (94) and RecO are recombination enzymes but their specific functions in the overall process have not been properly characterized. The RecQ helicase probably functions in the RecF pathway, and has been proposed by Hanada et al as a supressor of illegitimate recombination because several of its mutants have been shown to increase the rate of of this process from 20 to 300 fold (94).

b. In Eukaryotes: the mechanism of recombination in eukaryotes is much less understood than in *E.coli*, and the various enzymes, especially helicases that function in the process have not been fully characterized. However, the identification of structural homologues of the RecA protein in eukaryotic systems, such as the *S. cerevisiae* and human RAD51 proteins with respective molecular masses of 43kDa and 37kDa, suggests the possibility of a conserved mechanism in recombination from prokaryotes to eukaryotes (95,96). Both proteins exhibit DNA unwinding activity and their single stranded DNA binding properties recall the nucleo-protein filament described for the *E.coli* RecA protein (97,98). Sung P. has demonstrated that the *S. cerevisiae* RAD51 protein catalyzes the base pairing reaction and DNA strand-exchange between circular single-stranded Φ X174 or M13 and its respective homologous linear double stranded form (99,100).

Eukaryotic proteins with characteristics similar to those of the *E.coli* RuvC protein have been isolated from both calf thymus and CHO cells (101), and although these activities have not been characterized in detail and their mechanism/s of action are not yet defined, they may probably participate in reactions involved in the resolution of recombination intermediates in these systems.



RecA protein binds to single stranded DNA to form a nucleoprotein filament

This nucleoprotein filament invades a contiguous DNA in search of some homologous sequence

Annealing begins once homology is found and the hybrid region is extended

The hybrid is completed and a D loop is formed

Figure 2.3.1: Model of the activity of RecA protein in recombination in E.coli.

a) formation of holliday junction



b) extension of heteroduplex region

RuvAB

RecG (Holliday junction specific helicase)

c) resolution of junction

RuvC

(Holliday junction specific endonuclease)

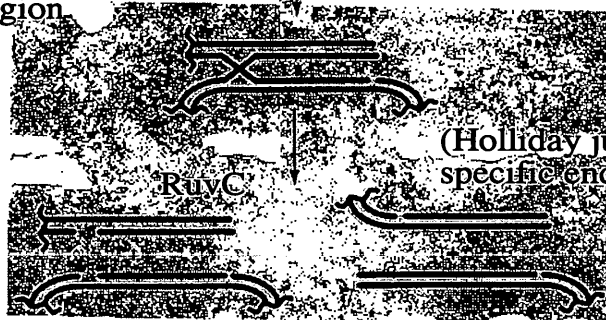


Figure 2.3.2. Resolution of Holliday junction:
The resolution of Holliday junction, to yield mature recombinant molecules is operated by junction-specific enzymes.

Homologous recombination in all cells implies correct base pairing and DNA strand-exchange between homologous DNA sequences. A special type of recombination, the site-specific recombination, brings together and joins different segments of DNA from different genes and often results in the re-arrangement of these sequences. A good example of *E.coli* site-specific recombination in prokaryotes is the integration of the bacteriophage λ in the genome. Integration of λ in the bacterial genome requires the phage protein integrase, which directs the recombination process between two specific sites containing short sequence homology between the two DNA molecules (102,103). In higher eukaryotes, the most characterized example of site-specific recombination is the V(D)J recombination in which mature immunoglobulin and T cell receptor genes are assembled in maturing lymphocytes (104), and involves the cleavage and subsequent re-joining of the "V", "J" and "D" gene fragments. V(D)J recombination takes place in developing lymphocytes and is triggered by the action of two adjacent unrelated genes designated RAG-1 and RAG-2 (105,106) since the co-expression of these genes in non-lymphoid cells confers, to such cell lines, the ability to perform V(D)J recombination. V(D)J recombination requires the introduction of double strand breaks (DSBs) in specific sites on the DNA, called recombination signal sequences (RSS), between the heptamer signal sequence and the first nucleotide of the adjacent coding sequence (107). This cleavage action generates various gene fragments called signal ends and coding ends, which are reaction intermediates and are characterized by having two different types of DNA termini. The signal ends join to each other, in a head to head manner, to form the signal joins and likewise the coding ends join to form the coding joins. Joining of the coding ends to form the coding joins is usually preceded by some degree of base loss or base addition to the ends, termed processing, whereas joining of the signal ends does not necessitate processing. Successive fusion of the variable, joining and diversity segments yields a complete gene, and owing to the presence of multiple segments of each type, the many possible combinations between these segments generates the high diversity of antigen receptor sequences (107).

V(D)J recombination initiates with the introduction of double strand breaks (DSBs) on the DNA molecule similar to those caused by ionizing irradiation. It was, therefore conceivable that a common mechanism existed for both cellular processes, and this was further strengthened by the finding that *scid* mice, defective for V(D)J recombination, were hypersensitive to X-ray irradiation and were also defective for the repair of double strand breaks (DSBs) (108-110). Search for the genes that could complement these recombination and repair defects resulted in the isolation of the mammalian genes XRCC5 and XRCC7 encoding, respectively, for the 83kDa subunit of the Ku antigen and the catalytic subunit of the DNA-dependent protein kinase PK350. This finding suggested the involvement of these two proteins in both the repair of double strand breaks and V(D)J recombination (111-114). The involvement of DNA-PK in the repair of double strand breaks and V(D)J recombination has also been documented by Blunt et al (115). These authors, in fact, showed that the

deficiency in performing V(D)J recombination and DSB repair also showed in the V3 mutant hamster cells was a result of defects in DNA-PK_{cs} activity, and brought evidence that yeast artificial chromosomes containing the DNA-PK_{cs} gene could complement both deficiencies in these cells. The Ku protein is an abundant nuclear two-subunit protein (83-kDa and 70-kDa) that binds dsDNA termini irrespective of the DNA sequence or structure. It binds DNA in multiple copies and slides along the molecule, without energy requirement, to form a structure like beads on a string with a 25-bp periodicity. Mutation in the XRCC5 gene impairs the formation of both the signal and coding joints in V(D)J recombination (115), suggesting that the Ku protein, by virtue of its DNA end-binding properties, directly mediates the formation of these intermediates. Likewise mutation in the XRCC7 gene causes an accumulation of the hairpin structures typical of recombination coding ends, suggesting that this protein is involved in processing the coding ends to form coding joints.

Tuteja et al (116) have described the ATP-dependent DNA unwinding as well as the ssDNA-dependent ATPase activities of the Ku protein, and these two activities have been shown to be required both in V(D)J recombination and the repair of DNA double strand breaks. A model for the involvement of Ku in DSB repair, proposed by Roth, D.B. et al (108), suggested that the Ku protein held DNA ends together for the repair reactions to occur. Alternatively, the Ku protein, in conjunction with DNA-PK could be involved in regulating the assembly of the multi-protein complex involved in DSB repair. Jackson, S.P. et al have also proposed a model for the involvement of the Ku protein in V(D)J recombination. According to this model, the helicase activity of Ku would serve to open the hairpin structures of the coding ends and render them susceptible to the single stranded DNA nuclease activity by which these ends are processed to form the coding joints (114).

2.4. Helicases and DNA Replication

In all organisms, DNA replication is the central event in the cell-cycle and usually leads to cell division. The faithful transmission of the genetic information from one generation of cells to another depends on the accuracy of replication since any error in this process often leads to mutations in the DNA molecule.

Whereas all other metabolic processes generally involve the interaction of the specific cellular factors required in such processes with the relative segments of the DNA, replication involves the entire DNA contained in a cell. Replication, however, is also the only DNA metabolic process restricted to only one phase of the cell-cycle: the S phase, and must also occur only once in a cycle (117). Put together, these features of the replication process

indicate it as the DNA metabolic process requiring the most extensive unwinding of DNA by helicases within a limited time interval and suggest that enzymes involved in replication, and therefore also replicative helicases, must possess peculiar properties that clearly distinguish them from other enzymes of their own kind. However, for all their supposed importance, helicases that function in replication are very poorly understood and their possible mechanism of action, in eukaryotes, is yet to be elucidated. This discrepancy may be attributed to the complexity of the replication process or to the complexity of the various interactions between the various classes of enzymes that play important roles in replication. It is also possible that replicative helicases have relatively short biological half-lives, since they function only during the S-phase, thus rendering their isolation and characterization a very difficult task. In fact only very few isolated eukaryotic DNA helicases have been clearly shown to take part in replication in eukaryotes.

DNA replication may be divided into three phases, namely, the initiation phase, the elongation and the termination phase. Each phase distinguishes itself from the others by the peculiar conformation of the DNA template, and by the nature of the various enzymes involved.

Initiation of replication is the process by which the specific enzymes are assembled on the DNA template at or near the replication origin to form the initiation complex. This usually starts with the recognition and binding of the origin sequence (also called the *Ori*) by a specific DNA binding protein: the initiator protein. The requirements for accurate initiation of DNA replication may vary between various species and may reflect the necessity to regulate replication at the initiation stage.

The elongation phase is a continuation of the initiation process and requires the concerted action of different classes of enzymes among which are helicases, primases, polymerases, ligases and single stranded DNA binding proteins. With the recruitment of processivity factors, the initiation complex is transformed into the replication complex: the helicase unwinds the DNA template in conjunction with the gyrase and SSB; the primase synthesizes primers which are elongated by the polymerase. The elongation of the DNA strand is a continuous process on the leading strand but discontinuous on the lagging strand and, therefore, requires the action of other enzymes that remove the RNA primers and those that seal these short segments into a continuous strand. DNA synthesis on the lagging strand also requires fresh recruitment of the primase for the synthesis of each new Okazaki fragment.

Termination of DNA replication involves the dis-assembly of the replication complex with consequent release of the daughter DNA molecules, and may require the action of a specific termination protein. If it can be said that the initiation and the elongation phases of DNA replication require the unwinding of double stranded DNA to generate the single

stranded template and, therefore the action of helicases, it must be said that replication termination implies inhibition of helicase-catalyzed DNA unwinding (118-120).

a. in *E.coli*. at least two DNA helicases are located at each fork of chromosomal DNA replication in *E.coli*: the DnaB helicase, a 5' to 3' translocating enzyme and PriA helicase which moves in the 3' to 5' direction along the bound DNA strand (32). Other proteins involved in replication in *E.coli* include DnaA, the initiator protein; DnaC which forms a complex with DnaB and serves to deliver the helicase to the DNA template; DnaG, the primase; SSB, involved in stabilizing the single stranded DNA template during synthesis of the lagging strand; DNA polymerase III holoenzyme, a multi-protein complex responsible for DNA synthesis on both the leading and lagging strands; Rnase H, responsible for the removal of the RNA primers on the lagging strand; the replication termination protein and Topo IV, the gyrase which separates the daughter DNA molecules at the termination of replication. A model which shows the organization of some of these factors at an *E.coli* replication fork can be seen in figure 1.6.3 in the section on the mechanism of DNA unwinding by helicases.

The Rep helicase is another important replicative helicase of the *E.coli* required for the rolling-cycle replication of the duplex replicative form (RF) of a number of ssDNA phages such as Φ X174 and M13 DNA. This enzyme has a denatured molecular mass of 65 kDa and translocates in the 3' to 5' direction along the bound DNA strand. After cleavage at the origin of replication by the initiator protein (gpA for Φ X174 and gp2 for M13) to generate the free 3'-OH for extension by DNA polymerase III, the Rep protein melts the duplex replicative form and displaces the cleaved strand in its 3' to 5' translocation. This enzyme efficiently hydrolyzes ATP and dATP, as energy source for the unwinding reaction, while GTP and dGTP are hydrolyzed one-third as rapidly and other nucleotides are poorly hydrolyzed. Mutations in the Rep locus are not lethal for the host cell, but block RF replication of phages, and cause slower fork movement (1). Studies on the mode of interaction of the Rep protein with DNA have led to the proposal of the two model mechanisms, the "rolling" and the "creeping" models, of helicase-catalyzed DNA unwinding.

Initiation of DNA replication in *E.coli* generally occurs at a unique and specific sequence called *ori C* (121,122). This 245-bp sequence contains some important elements such as four 9-bp binding sites for the initiator protein and three 13-bp sequences rich in A and T from where effective DNA strand separation, in the origin, begins. The first step in origin activation is the formation of a complex between DnaA and ATP, followed by the co-operative binding of 10-20 units of this complex to the DNA in the region of the origin. It has been shown that only the DnaA-ATP complex is the active form of this enzyme in the initiation of DNA replication in *E.coli* (121) and, according to Bramhill and Kornberg (123), this complex promotes the opening of the DNA in the region of the 13-mers, apparently without energy requirement since it does not require hydrolysis of the ATP bound to the enzyme. The origin unwound by the binding of the DnaA-ATP complex is a suitable binding

substrate for the helicase, DnaB. However, since this helicase has low affinity for both single stranded and double stranded DNA it needs to be transferred to the exposed ssDNA template by the concerted action of DnaA and DnaC. Winker and Hurwitz (124) have shown that, in the presence of ATP, DnaB forms a complex with DnaC, and that via the latter, the complex recognizes DnaA already bound to the origin which, therefore, helps to load the helicase onto the template while DnaC is released (124). The dissociation of DnaC from its complex with DnaB seems to activate the helicase for its later activity of ATP-dependent DNA unwinding in the presence of the gyrase and single strand binding proteins (125,126). The unwinding action of DnaB and *E.coli* DNA replication from *oriC* have been shown to be bi-directional both *in vivo* and *in vitro* (127). Therefore, this fact implies that two complexes of DnaB must be loaded during the activation of the origin, one on each strand of the DNA template. Once DNA unwinding is begun, the primase synthesizes RNA primers and these are subsequently elongated by the DNA polymerase III holoenzyme (127), itself dimeric at the fork to guarantee simultaneous synthesis of both the leading and lagging strands.

DnaB is a protein of 50kDa but its active conformation, in replication, is that of a hexamer, a conformation which reflects the oligomeric nature observed for some helicases. Its polarity of translocation is 5' to 3', indicating that the enzyme interacts directly with the DNA lagging strand. Dna B efficiently utilizes also CTP and GTP besides ATP as energy sources for the unwinding reaction. The helicase also interacts with, and directs the rest of the replication enzymes to the template, and in doing so, acts as a link between the cellular initiation factors and those involved in elongation. It helps to recruit DnaG, the primase, to the lagging strand and then activates it to synthesize multiple primers necessary for the discontinuous synthesis of Okazaki fragments. Tougu et al showed that DnaB interacts physically with the primase since DnaG mutated at the site of interaction with DnaB could not be recruited to the replication fork (128). The mechanism of activation of the primase by DnaB is not very clear, but the helicase is believed to generate secondary structures, on the DNA template, which the primase may recognize. The discontinuous nature of primer synthesis on the lagging strand implies that DnaG is released from the template after each priming event and re-recruited for each round of priming and synthesis of Okazaki fragment.

Single stranded DNA binding proteins (SSBs) play an essential role in DNA replication by stabilizing the unwound DNA strands and by melting DNA secondary structures that may impede the progression of the polymerase. Such stabilizing action of these proteins on DNA single strands often results in a stimulatory effect on the activity of enzymes such as helicases (129) or DNA polymerases (130) that interact with or require single stranded DNA. Not surprisingly, complexes between SSB and these enzymes have often been reported. According to Mock et al (131), however, the polymerase III holoenzyme advances closely behind the helicase during replication in *E.coli*. Therefore stabilization of ssDNA, by SSB during this process, may be required only for the discontinuous synthesis of Okazaki fragments on the lagging strand.

The multi-component DNA polymerase III holoenzyme synthesizes both DNA strands by elongating the RNA primers synthesized by DnaG. DNA synthesis is continuous on the leading strand but discontinuous on the lagging strand, and this implies multiple dissociations of the polymerase from the DNA template and re-associations to it during synthesis on this strand. The core enzyme of polymerase III consists of the α subunit (the 140-kDa polymerase), the ϵ subunit (the 23-kDa proof-reading exonuclease), and the θ subunit. Although this core enzyme can successfully synthesize DNA strands, it has been shown that processive synthesis of DNA requires the action of the other components of the holoenzyme (132). Other enzymatic activities are also required to convert the DNA segments synthesized on the lagging strand into a continuous DNA strand. These are: RNase H which removes the RNA primers to allow for gap-filling DNA synthesis; DNA polymerase I which fills the gaps created by the nuclease activity of RNase H and the ligase, responsible for sealing the nicks to generate a continuous DNA strand.

Termination of DNA replication, during bi-directional replication in all cells, may simply occur when two replication forks advancing in opposite directions meet. However, the presence of a large termination zone which is located diametrically opposite *ori C*, in the *E.coli* chromosome has also been reported (133,134). This termination zone contains some specific sequences capable of blocking the progression of replication forks. Termination of DNA replication in *E.coli* has also been shown to require the action of site-specific binding proteins, the *ter* binding proteins (TBP). These proteins bind to the specific sites contained in the termination zone (the *ter* sites) and form a stable DNA-protein complex which has been shown to inhibit DNA helicases such as DnaB and PriA (135, 136). Separation of the daughter DNA molecules is accomplished by topoisomerase at the end of the replication process.

b. in eukaryotes: replication in eukaryotes is much more complex than in prokaryotes and knowledge of the enzymes involved in eukaryotic DNA replication is less complete. Except in lower eukaryotes, helicases involved in DNA replication have not been unambiguously identified although many enzymes endowed with biochemical properties similar to bacterial replicative helicases have been proposed as probable candidates (137-139). However, as in prokaryotes, replication in eukaryotes can be divided into the phases of initiation, elongation and termination. As I mentioned earlier, significant differences between replication in prokaryotes and in eukaryotes, are found essentially in the initiation process. Contrary to the single origin of replication contained in the *E.coli* chromosome, eukaryotic cells contain several thousands of origins where DNA replication may start asynchronously. Eukaryotic origins are believed to be DNA structural elements containing A-T rich sequences. The presence of such large number of replication origins in eukaryotic cells is probably justified by the enormous size of the eukaryotic chromosomal DNA (compared to

that of *E.coli*) which must be replicated only once during the S-phase of the cell cycle. This, therefore, represents a major difference between the replication process in prokaryotes and eukaryotes since initiation from these multiple sites must be co-ordinated in order to allow all the DNA to be replicated while at the same time avoiding re-replication of any portion of the DNA during the same cell cycle.

Initiation of replication from a given eukaryotic origin requires the binding of the origin recognition complex (ORC) to form the initiation complex, as the first step in a series of events that lead to the activation of the origin. The formation of the initiation complex induces structural deformations in the origin-flanking region and causes a local melting of the origin to allow access for the replication proteins and machinery. However, due to the presence of numerous origins in the eukaryotic cell, and the requirement that DNA replication initiates only once in a cell cycle from an origin, the concept of replication licencing was introduced as a process by which eukaryotic cells regulate DNA replication. Licencing of DNA replication requires that once replication has initiated from an origin (called also firing of the origin) this origin can no longer be recognized by the initiation complex, and therefore can not initiate another round of replication, during the same cell cycle. Based on their study on the regulation of DNA replication in xenopus eggs, Leno, G. and Laskey, R. suggested that the integrity of the nuclear membrane was connected with licencing of replication in eukaryotes (140,141). The nuclear membrane in eukaryotes is its principal distinguishing feature from that of prokaryotes, and since the breakdown of this membrane occurs uniquely in mitosis, it represents a physical barrier to the nuclear import of cytosolic factors involved in licencing of replication. Today, it is widely held that a group of six eukaryotic proteins, termed minichromosome maintainance proteins (MCMs), which have been shown to be components of the replication initiation complex are involved in the licencing of DNA replication in eukaryotes (142,143). The process of replication licencing is, however, believed to involve two groups of cellular factors namely, the RLF-M, comprising the MCM group of proteins, and the RLF-B, a hitherto unidentified enzyme (or group of enzymes)(144). Ishimi, P. (44) has described a non-processive helicase activity associated with the MCM complex of proteins, which results the first documented evidence of the involvement of helicases in origin activation in higher eukaryotic systems.

The main DNA helicase shown to be involved in DNA replication, in the yeast *S. cerevisiae*, is DNA2. It translocates in the 3' to 5' direction along the DNA, has an Mr of 172 kDa and uses ATP and dATP as co-factors in the reaction of DNA unwinding (37). The mechanism of action of DNA2 is not clear, but its requirement for a fork-like structure in the substrate suggests its involvement in the elongation rather than in the initiation phase of DNA replication. Another yeast DNA helicase, helicase III, has also been proposed to be involved in DNA replication (145). Helicase III has a molecular weight of 120 kDa and uses ATP and dATP as DNA unwinding co-factors. It requires a single strand stretch of DNA for its

activity. The mechanism of action of helicase III is not known but its 5' to 3' polarity of translocation indicates that its enzymatic activity is synergic with that of DNA2.

Another significant difference in replication between prokaryotes and eukaryotes is in the nature and composition of the DNA polymerizing enzyme. Infact, prokaryotes contain only one principal replicative polymerase (DNA polymerase III holoenzyme in *E.coli*) that must function and synthesize DNA on both strands of a replication fork, whereas in eukaryotes, DNA synthesis on the two strands is shared between three polymerases: pol- α , pol- δ and pol- ϵ . In prokaryotes the polymerase needs to be free of the primase while synthesizing DNA on the leading strand since priming of this strand occurs only once in one cycle of replication, and for the discontinuous synthesis of Okazaki fragments on the lagging strand the primase is recruited anew, to interact with the polymerase, for each priming event. In eukaryotes, on the other hand, DNA polymerase α forms a complex with the primase, (DNA polymerase α -primase complex), involved in the priming event and the synthesis of the initiator DNA on both strands, while processive DNA synthesis is carried out by pol- δ and pol- ϵ . Therefore, eukaryotes have developed a polymerase switching mechanism that involves the pol α -primase complex in the priming of DNA replication at the origin of replication as well as for the synthesis of every Okazaki fragment, and then a second polymerase which is not associated with the primase (pol- δ or pol- ϵ) to complete the DNA synthesis on both strands.

2.5. Replicative Viral DNA Helicases

The known role of helicases in the replication of the DNA of some animal viruses such as the Simian Virus 40, Herpes simplex virus and papilloma virus has helped in the elucidation of the mechanism of chromosomal DNA replication. Table 2.5.I presents a list of some replicative viral DNA helicases with some of their properties.

Table 2.5.I. Properties of some viral DNA helicases

Helicase	Mr (kDa)	Direction of unwinding	NTP cofactor	Function
SV40 large T antigen	82	3' to 5'	ATP, dATP, dTTP, >UTP, dCTP	replication
Polyoma virus T antigen	88	3' to 5'	ATP, dATP, >CTP, UTP.	replication
HSV UL5, UL8, UL52	120/97/70	3' to 5'	ATP, GTP	replication
HSV UL9	94	3' to 5'	ATP, CTP, dATP, dCTP, UTP	replication
BPV E1	72	3' to 5'	ATP, dATP, CTP, dCTP, UTP, dTTP, GTP, dGTP	replication

2.5.1. The Simian Virus 40 large T. Antigen

Studies on DNA replication in the Simian virus 40 and the identification of the various proteins involved in this process have proved the most useful tools for the study of the mammalian process since viral DNA is replicated by the host replication machinery together with one single viral protein, the viral large tumour antigen. Therefore the development of a cell-free system and the use of purified proteins, for the study of DNA replication in SV 40, has allowed the identification of proteins and factors involved in mammalian DNA replication.

The SV 40 large T antigen is a multi-functional phospho-protein involved in both the initiation and elongation steps of viral DNA replication. Monomeric T antigen is a protein of

708 amino acids with a molecular mass of 82 kDa endowed with a 3' to 5' processive DNA unwinding activity. However the helicase-active form of the enzyme has been shown to be that of a hexamer, in analogy to the hexameric nature of other helicases such as the *E.coli* DnaB and the rho protein (146). During origin denaturation, T antigen binds specifically and with high affinity to a palindromic sequence in the viral origin of replication, and in the presence of ATP and Mg^{2+} , assembles into a double hexamer that covers the region of the core origin (146,147). This double hexamer-DNA complex induces structural deformation in the bound DNA and causes the melting of the region of the origin, and therefore catalyzes the initial non-processive unwinding of the viral origin of replication. The assembly of the T antigen double hexamer and the melting of the origin by the T antigen is modulated by the state of phosphorylation of the enzyme. In-fact, it has been shown that the activity of origin denaturation is inhibited by phosphorylation of the serine residues at amino acid positions 120 and 123 (148,149) of the enzyme. The exact mechanism by which phosphorylation at these sites inhibits initial origin unwinding by T antigen is not known. However, since the assembly of the T antigen double hexamer at the viral origin is impaired in this state of phosphorylation, but the uni-directional helicase activity of the T antigen hexamer at a single replication fork is not affected, it is possible that the affinity of the enzyme for the viral origin is altered by phosphorylation at these sites.

Once the origin of replication is melted and unwound, the host replication protein A (RPA) binds to both strands while a topoisomerase releases the torsional stress generated by the unwinding. DNA polymerase α -primase synthesizes RNA primers on both strands and further extends them by synthesizing the short segment of the initiator DNA (iDNA). A physical association between T antigen and DNA polymerase α -primase has been detected and it seems clear that this functional interaction between the two proteins plays an important role in the initiation of DNA synthesis. In fact, modulation of such interaction, either by phosphorylation or through protein-protein interaction, could provide a mechanism for regulating DNA replication. In this context it is interesting to mention that the anti-oncogene protein p53 competes with DNA polymerase α -primase for binding to T antigen and that binding to p53 inhibits the activities of T antigen in viral DNA replication (15,151). Interaction of T antigen with DNA polymerase α -primase complex stimulates both primer synthesis and its subsequent elongation by the primase.

The SV40 large T antigen helicase is also the only viral protein involved in the processive unwinding of the DNA throughout the replication process. In fact after the initial origin opening and the priming event, the host replication factor C (RF-C) binds to the 3' end of the primer/template junction and loads the proliferating cell nuclear antigen (PCNA), displacing the polymerase α -primase complex and recruiting polymerase δ (polymerase switching). Polymerase δ is the enzyme responsible for DNA synthesis on both the leading and lagging strands. Synthesis is continuous on the leading strand but discontinuous on the lagging strand. This discontinuous nature of the synthesis of Okazaki fragments may need

the activity of polymerase ϵ besides pol δ and also requires fresh recruitment of the polymerase α -primase complex for each priming event. This situation can be seen more clearly in figure 2.5.1.

As I mentioned earlier the basic helicase form, which constitutes a DNA unwinding centre, of the SV40 T antigen is the hexamer. However, using electron microscopy to study the intermediates of an unwinding reaction, Wessel et al. (30) observed that these reaction intermediates were made of two apparently different conformations. One type, representing about 75 % of the unwinding intermediates was present as an unfolded unwinding bubble with a large T antigen hexamer in the centre of each fork while SSB covered the exposed single stranded portions of the DNA. The other conformation was represented by the so called "rabbit ears"-containing structures, consisting of two loops of ssDNA covered with SSB which appeared to emerge from, and return to a bi-lobed T antigen double hexamer complex, sitting on the double-stranded DNA (figure 1.6.2).

Whereas in the unfolded intermediate, with each T antigen hexamer sitting on one replication fork, the helicase seemed to translocate on the DNA, the "rabbit ear" conformation seemed to represent a large helicase complex (the dodecamer) sitting on duplex DNA while threading the DNA molecule and unwinding the same as it transited through the centre of the double hexamer helicase. However, since the bi-lobed helicase complex is shown to interact simultaneously with the two replication forks, the unit unwinding centre was still the hexamer unit (30). Incubation of Large T antigen with the monoclonal antibody PAb101, prior to the unwinding reaction, favoured the formation of intermediates with the "rabbit ear" structures rather than the unfolded-conformation intermediate. Since antibodies bound to the T antigen simulate *in vivo* conditions in which the DNA-unwinding complex of the T antigen seems to be bound to structural elements such as the nuclear matrix, binding which might stabilize and stimulate the replication complex, this observation may indicate the possible *in vivo* mechanism of DNA unwinding by the SV 40 large tumour antigen.

2.5.2. Herpes Simplex Virus (HSV)

Herpes simplex virus 1 (HSV 1) encodes seven proteins that are required to initiate and sustain DNA replication from the viral origin of replication. These proteins are encoded by the UL5, UL8, UL9, UL42, UL52 genes, the ICP8 protein (infected cell polypeptide 8) and the DNA polymerase. Three of these proteins, the products of UL5, UL8 and UL52 genes form a heterotrimer that possesses both DNA helicase, ATPase and DNA primase activities.

Dodson et al (152) showed that a sub-assembly of this complex made of the UL5/UL52 subunits exhibits all the enzymatic activities of the trimer, and therefore forms the core enzyme, while the presence of the UL8 subunit, which has no detectable enzymatic or DNA binding activities, increases the rate of primer synthesis of the trimeric complex. Such increase in the rate of primer synthesis, due to the presence of UL8, also implies an increase in the efficiency of lagging strand synthesis (153). UL8 is also required for the synthesis of long stretches (> 20 kb) of single stranded DNA during the rolling cycle DNA replication and to reverse the inhibitory effects of ICP8 on the primase, the helicase and the ATPase activities of the UL5/UL52 complex observed at high SSB/template ratios (154).

Although the UL5 protein bears sequence homology to the NTP-binding domain of known helicases and possesses the conserved helicase motifs in its amino acid sequence, it does not show any helicase or ATPase activity unless it is in association with the UL52 protein. In contrast to this observation, a single amino acid mutation (aspartate to glutamine at amino acid position 628) in the UL52 subunit completely abolishes the ability of the UL5/UL52 complex to support the replication of an origin-containing plasmid *in vivo*, to support lagging strand synthesis and to synthesize oligoribonucleotides *in vitro*. Furthermore, partially purified preparations of UL52 alone have been shown to exhibit the primase activity of the UL5/UL52 complex, all of which indicate that the UL52 subunit alone may be endowed with this activity of the complex(155,156).

The UL9 protein is an origin sequence-specific binding protein and has also been shown to possess ATPase as well as a 3' to 5' DNA unwinding activity. The N-terminal portion of this protein contains the characteristic amino acid sequence motifs of DNA and RNA helicases while the C-terminal region is responsible for the sequence-specific binding activity. This origin binding activity of UL9 may serve to initiate the assembly of a multi-protein complex that eventually leads to the activation of the origin. This activity, however, is not required for the DNA unwinding activity of the protein (157).

The UL42 protein is a non specific double stranded DNA binding protein which has also been shown to interact with the DNA polymerase (158). It is not known, however, whether this interaction has any functional significance or whether the DNA binding activity is an essential component of the function of this protein in the replication of the Herpes simplex DNA.

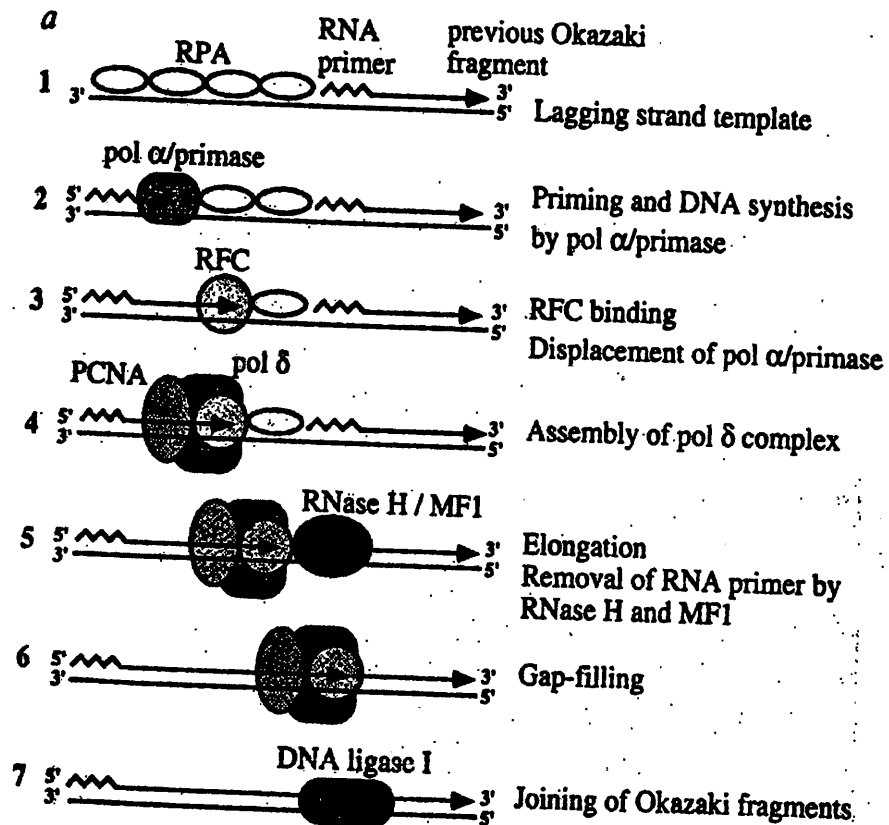


Figure 2.5.1: Mechanism of lagging-strand synthesis at a eucaryotic cell DNA replication fork. Model for a DNA polymerase switching mechanism during lagging-strand replication.

2.5.3. The Bovine Papilloma Virus

The bovine papilloma virus (BPV) genome encodes two proteins that are necessary to activate and sustain viral replication namely, the E1 and the E2 proteins. The E1 protein is a 72 kDa nuclear sequence-specific DNA binding protein that serves to recognize the viral origin, a feature which is typical of a replication initiator protein. This protein has been shown to bind the origin in different oligomeric forms and to possess a 3' to 5' DNA unwinding activity. In cell-free replication systems, the E1 protein binds to the origin by itself as a trimeric complex, albeit with low sequence-specificity, and is capable of localized melting and unwinding of the origin (159,160). The trimerization of this protein is induced by the DNA template and results in a complex linked topologically to the DNA with the three molecules of E1 forming a ring-like structure that encircles the DNA. The E1 protein is also the only viral protein required for the initiation of viral replication *in vitro* in a manner similar to that of the large T antigen in the replication of the SV40. The observed different forms of the E1 protein bound to the origin probably represent intermediates states in a functional transition of the E1 protein from a sequence-specific origin binding protein to a form of E1 competent for the initiation of viral DNA replication. The mechanism behind this transition is not very well understood, but it is believed that the activities of the protein may be altered by multimerization. The replication of papilloma virus *in vivo*, however, requires the co-operative interaction of the E1 protein with the viral transcription activator protein, the E2 protein. This difference between replication *in vitro* and *in vivo* is believed to be due to the limited sequence-specificity of the E1 binding to the viral origin *in vitro*.

The E2 protein is a 48 kDa sequence-specific transcription activator. It is also capable of independent binding to the viral origin, as a dimer, with low sequence specificity, but its interaction with the E1 protein raises the sequence-specificity binding of E1. The requirement of E2 in viral replication *in vivo* is absolute since no replication is detected in its absence, thus indicating that the E2 protein performs an essential function in viral replication *in vivo* (161). Such a function could be to serve as a specificity factor for E1. This requirement of E2 is also highly specific since no other viral transcription factors tested could stimulate the specificity of origin binding by the E1 protein in viral DNA replication. This property of the E2 protein supports the concept that transcription factors have a direct role in the initiation of DNA replication (162).

2.5.4. Human Papillomavirus (HPV)

The study of DNA replication in human papillomaviruses has not been as detailed as that of the bovine virus due to the lack of an *in vitro* system that supports viral replication.

Therefore, studies on the human virus have essentially focused on the aspects that draw some analogy with the bovine virus. For example, the human papillomavirus type 11 (HPV-11) has been shown to encode two proteins, the E1 and E2 proteins, necessary and sufficient to support viral replication together with the host replication machinery. The full length HPV E1 and E2 proteins have been expressed in insect cells for the study of replication in the HPV system. Both proteins form a heteromeric complex within the insect cell similar to the heteromeric complex of the BPV proteins, and both have been shown to localize to a DNA fragment containing the viral origin of replication. Although there has not been a detailed biochemical characterization of these proteins, the human papilloma virus type 11 E1 and E2 proteins share some amino acid homology with BPV E1 and E2 proteins, and also possess the same replication-associated activities of their corresponding bovine papillomavirus proteins, suggesting that the replication process may be conserved between BPV and HPV-11 (163).

The human papillomavirus type 11 E1 protein is a nuclear phosphoprotein with a molecular mass of 80 kDa. It possesses an ATPase and GTPase activity, and this activity is believed to be a component of a helicase activity predicted for this protein in analogy to the BPV E1 protein. Contrary to the BPV E1 protein, the human papillomavirus E1 protein does not show independent binding to DNA, but does so only when it is associated in the heterocomplex with the E2 protein.

The E2 protein is a nuclear sequence-specific DNA binding phosphoprotein with a molecular mass of 42.5 kDa. Its specific function in viral DNA replication seems that of targeting the E1 protein, when both are in the heterodimeric complex, to the viral origin of replication. In analogy to the E2 protein of BPV, the HPV-11 E2 protein is also a transcription activator. Computer analysis predicts extensive similarities in secondary structure among all the sequenced papillomavirus E2 proteins, and their ability to activate an enhancer region containing the canonical DNA-binding site (ACCN₆GGT) seems conserved since the viral E2 proteins are interchangeable between various papillomaviruses.

2.6. Other Eukaryotic DNA Helicases

The multiple interactions observed between DNA helicases and some cellular factors such as the single stranded DNA binding protein (SSB), Replication factor C (RF-C) and polymerases have been employed in various techniques to isolate these enzymes from several eukaryotic sources including calf thymus and mouse as well as human cells. These procedures have led to the isolation and characterization of several eukaryotic helicases co-purifying with various cellular factors, and such helicases have often been named after the factors with which they co-purified. Other purification procedures have immobilized various cellular factors on chromatography resins and used such resins as affinity matrices to isolate eukaryotic helicases. The apparent intentions of these purification techniques were to isolate

helicases for which *in vivo* functions could be tentatively deduced based on knowledge of the *in vivo* cellular processes in which the interacting factors also participate. For example, a helicase that co-purified with a polymerase, whether or not both proteins formed a stable complex with each other, might be expected to function in the same *in vivo* cellular process as the polymerase. Likewise a helicase isolated by affinity chromatography on a column with immobilized SSB might be involved in the DNA metabolic process requiring that particular SSB. A few examples of helicases isolated through these techniques are the following:

Helicase E was isolated from calf thymus and co-purified through many chromatographic steps with polymerase ϵ from which it took its name. It consists of a single polypeptide chain of 105-kDa and translocates in the 3' to 5' direction along the bound DNA strand. An involvement in DNA repair and recombination has been suggested, together with polymerase ϵ , for this helicase (164).

Helicase F was also isolated from calf thymus and co-purified with the replication protein A (RPA) up to the last purification step. This enzyme moves in the 5' to 3' direction with a monomer mass of 72-kDa but was shown to exist also in an oligomeric form. Since the presence of RPA stimulated the processivity of helicase F, the interaction between these two proteins was thought to be functionally relevant. Helicase F has been suggested to be involved in DNA replication (137).

DNA helicase ϵ , isolated from HeLa cells, was not purified through its interaction with other cellular factors but its DNA unwinding activity was shown to depend nearly entirely on the presence of the human single stranded DNA binding protein. This enzyme was shown to translocate in the 3' to 5' direction and possessed the ability to unwind blunt end substrates. It has been proposed to function in DNA replication (138).

By affinity chromatography on RPA-bound resin, a DNA helicase was purified from mouse FM3A cells in an effort to isolate an enzyme that interacted with this single strand binding protein. This enzyme which consisted of two polypeptides of 21-kDa and 28-kDa subunits was shown to translocate in the 5' to 3' direction and its processivity seemed to be enhanced by SSB. It is thought to be involved in DNA replication (139).

These methods of enzyme purification and the consequent hypotheses of *in vivo* functions appear to offer a convenient means of overcoming the difficulty in assigning roles to isolated eukaryotic DNA helicases. However, such efforts must be corroborated by strong experimental evidence documenting the involvement of a particular helicase in a given DNA metabolic process.

2.7. DNA Helicases of HeLa Cells

A systematic study of DNA helicases contained in HeLa cell nuclei has led to the isolation and characterization of six different molecular species in our laboratory named (Human DNA Helicases, HDHs) HDH I, HDH II, HDH III, HDH IV, HDH V, HDH VI (165-171) and two recently characterised ones namely HDH VII and HDH VIII. The genes for two of these enzymes have been identified and some of their *in vivo* functions have also been determined. A detailed flow-chart of the general purification scheme of these human DNA helicases from HeLa cells is shown in figure 2.7.1 while some of their individual biochemical properties, outlined in table 2.7.1 will be summarized here.

Human DNA helicase I (HDH I) is a 65 kDa protein as determined by SDS-PAGE, but the molecular weight in native conditions has not been estimated. Its relative abundance is 0.11 mg in 100g of HeLa cells, and it exhibits a 3' to 5' polarity of translocation while unwinding duplex DNA. HDH I requires a single stranded portion of more than 11 bases, it can unwind duplex regions up to 45 base pairs, and its activity is not influenced by the presence or absence of hanging tails in the substrate. HDH I also catalyses the ATP-dependent unwinding of DNA/RNA hybrids (165).

Human DNA helicase II (HDH II) was initially identified by Csordas-Toth et al as a protein interacting with a human DNA region containing an origin of replication and shown, by N-terminal sequence analysis, to be identical to the Ku antigen (166) but its DNA unwinding properties were determined later (116). HDH II/Ku moves in the 3' to 5' direction along the bound DNA strand and the helicase activity is remarkably influenced by the presence of a fork-like structure in the substrate. Ku is a heterodimer of 70 and 83 kDa subunits, data further confirmed by its native molecular weight of about 155-kDa. The two subunits of HDH II/Ku can not be separated by any physical methods, not even in the presence of salt concentrations up to 2 M, and notwithstanding the fact that the genes encoding the subunits have been localized on different chromosomes. Although both subunits contain cystein residues, they do not seem to be held together by di-sulphide bridges in the heterodimer. HDH II/Ku is an autoantigen recognized by sera of scleroderma pigmentosum and lupus erythematosus patients. It binds to the termini of duplex DNA and then slides along the molecule in several copies, without energy requirement, to form a structure like beads on a string with a 25 bp periodicity. Due to the availability of cDNA constructs containing the genes for the separate subunits of HDH II in an expression vector, I expressed these separate subunits in an effort to dissect the *in vitro* properties of the HDH II/Ku heterodimer.

The properties of these recombinant separate subunits will be described in detail in chapter 4.2 of this thesis.

Table 2.7.I. Summary of properties of HDH I - HDH VI

Property	HDH I	HDH II	HDH III	HDH IV	HDH V	HDH VI
Abundance (mg/100g of cells)	0.11	0.078	0.085	0.070	0.004	0.134
Size kDa (SDS-PAGE)	65	70/83	46	100	92	128
Size kDa (Native)	N.D	158	N.D	N.D	N.D	128
Sedimentation coeff.	N.D	7.45	N.D	N.D	N.D	6.75
Stokes radius (Å)	N.D	~ 46	N.D	N.D	N.D	~ 44
Direction of unwinding	3' to 5'	3' to 5'	3' to 5'	3' to 5'	3' to 5'	3' to 5'
DNA-RNA unwinding*	N.D	-	-	+	-	-
RNA-DNA unwinding*	+	-	N.D	+	N.D	N.D
RNA-RNA unwinding*	N.D	-	-	+	N.D	-
Req. fork-like structure	-	+	+	-	-	+
Req. of ssDNA (bases)	> 11	> 84	> 84	> 84	< 49	< 84
Max. unwinding (bp)	> 40	> 40	> 40	< 40	< 40	< 40
ssDNA-dep. ATPase	+	+	+	+	+	+
Phosphorylation by cdc2	+	-	+	+	N.D	N.D
Phosph. by CKII	+	-	+	+	N.D	N.D
Gene identified as	N.D	Ku	N.D	Nucleolin	N.D	N.D
Mapping	N.D	70: 22q13 83:2q33-35	N.D	2q12-qter	N.D	N.D
Function	N.D	**	N.D	***	N.D	N.D

* DNA-RNA is a short RNA stretch annealed to a long DNA strand, RNA-DNA is the opposite

** V(D)J recombination, X-ray dsDNA damage repair; *** Preribosome assembly; N.D Not Determined

Human DNA helicase III (HDH III) has an apparent molecular weight of 46 kDa as determined in denaturing conditions. It moves in the 3' to 5' direction and requires a fork-like structure in order to exert its helicase activity. The optimal concentration of ATP, equal to that of MgCl₂ for the DNA unwinding activity, is 1.5 mM. The native molecular weight of HDH III has not been determined (167).

Human DNA helicase IV (HDH IV) has a molecular weight of 100 kDa and possesses a 3' to 5' polarity of translocation. HDH IV does not require a fork-like structure in the substrate but can unwind only short duplex regions. HDH IV, later identified as human nucleolin (168), also unwinds RNA duplexes and RNA-DNA hybrids, and its unwinding activity is stimulated, in an additive manner, upon phosphorylation by cdc2 and casein kinase.

HDH V has a Mr of 92-kDa and seems to be present in very low amounts in the cell (only 0.012 mg were recovered from 300g of HeLa cells). However, the enzyme shows a very high specific activity and translocates in the 3' to 5' direction along the DNA.(169).

Human DNA helicase VI is a monomer of 128 kDa as determined both in native and under denaturing conditions. It cannot unwind DNA/RNA and RNA/RNA substrates, and it prefers a fork-like structure in a manner similar to HDH II and HDH III. Its catalytic properties are very similar to those of HDH III, but rabbit polyclonal antibody raised against HDH III was unable to recognise HDH VI in Western blotting (170).

HDH VII is the most recent addition to the family of DNA helicases characterized in our laboratory, and possesses the highest specific activity among all the enzymes so far described (see table 4.1.3). Among these helicases only HDH V possesses a specific activity comparable to that of HDH VII. The biochemical properties observed for HDH VII *in vitro* are outlined in more detail in chapter 4.1 of this thesis.

HDH VIII is another new member of the HeLa nuclear helicases isolated in our laboratory and exhibits a 5' to 3' polarity of unwinding. Its is a low abundant enzyme since only 0.100 mg was recovered from 300 g of cells. HDH III can unwind RNA duplexes as well as RNA/DNA hybrids. Amino acid sequence analysis of peptide fragments from a tryptic digestion of HDH VIII has shown that the enzyme is identical to the G3BP protein, an element of the Ras signal transduction pathway (171).

2.8. Human Disorders Associated with a Defect in DNA Helicases

Several human diseases (Xeroderma pigmentosum, Cockayne's syndrome, Bloom's syndrome and Werner's syndrome) are caused by defects in DNA helicases. Some human genes encoding proteins which belong to helicase families have been implicated in the process of DNA repair and mutations in three of them, XP-D, XP-B and ERCC6 have been identified in the human clinical syndromes known as Xeroderma pigmentosum and Cockayne syndrome. The clinical manifestations of these genetic disorders as well as the impaired DNA metabolic processes which give rise to their insurgence have been described in the section on the nucleotide excision repair mechanism (NER). Two other syndromes connected to helicases are the Bloom syndrome and the Werner syndrome.

2.8.1. Bloom's Syndrome (BLM)

Bloom's syndrome, an autosomal recessive genetic disorder, involves severe prenatal and postnatal growth deficiency (the average birthweight is a mere 1.9 kg and adult height 145 cm) as well as a predisposition to different types of malignant and benign tumours, variable immune deficiencies, skin pigmentation abnormalities and a complex behavioural pattern (14).

In individuals suffering from Bloom syndrome, breakage of metaphase chromosome is frequently observed as well as a spontaneous ten-fold increase in the rate of exchange between sister chromatids. The gene responsible for Bloom syndrome has been isolated and sequenced (172). The predicted gene product is a protein of 1417 amino acids with a molecular weight 159 kDa and contains the seven amino acid sequence motifs characteristic of helicases. This protein also shows a certain degree of homology to a family of DNA and RNA helicases: 42 % homology to *E.coli* Rec Q helicase, 43 % homology to the yeast Sgs1 protein and 44 % homology to human RecQL (173).

The role of RecQ helicase in the *E.coli* seems to be that of providing the necessary ssDNA fragment for the RecA protein in a manner similar to that of the RecBCD complex. Mutations in the Sgs1 gene, (the yeast homologue of the Bloom's syndrome gene product) induce slow growth, poor sporulation, missegregation in meiosis and an elevated recombination frequency in this species. Sgs 1 protein also interacts with topoisomerase II (174) and therefore may function in chromosome separation. The predicted size of Sgs1 (1447 residues) is similar to that of the Bloom syndrome gene product (BLM) and the two peptides also have similar base compositions outside of the helicase domains. Some

functional connection between BLM and topoisomerase II has been suggested based on the observations of Heartlein (175) that topoisomerase activity is decreased in BrdU-treated BS cells.

Contrary to what is observed in Xeroderma pigmentosum and Cockayne syndrome patients, DNA damage repair is not impaired in Bloom syndrome patients suggesting that the Bloom syndrome helicase is involved in some DNA metabolic process other than damage repair.

2.8.2. Werner's Syndrome (WS)

Werner's syndrome is a rare autosomal recessive disorder that mimics some of the characteristics of natural ageing. WS patients prematurely develop different age-related diseases such as type II diabetes mellitus, several forms of arteriosclerosis, malignant neoplasm, osteoporosis and ocular cataracts. These patients also manifest early graying and loss of hair, skin atrophy and generally aged appearance. Cell culture studies showed that the life-span of fibroblasts from WS patients was much reduced and was comparable to the life-span of fibroblast taken from more elderly individuals (176).

The gene responsible for this disorder has recently been identified as a helicase of the RecQ family (177). The predicted WRN gene product is a protein ~ 64 % identical to the human RecQL helicase, to the *E.coli* RecQ helicase and also to the yeast Sgs1 protein. As a putative helicase, the WRN gene product could be involved in different aspects of DNA metabolism. Proof of this is the elevated rate of mutation at specific genes, the elevated rates of non-homologous recombination, chromosomal instability and possible altered DNA replication (178).

The mechanisms by which these different diseases develop in WS patients are not well understood but, since DNA metabolism is altered in WS manifestations, it provides evidence that at least some component of "normal" ageing in normal individuals may be related to aberrations in DNA metabolism.

2.9. OBJECTIVE OF THE PRESENT WORK

DNA helicases catalyze an important step in virtually all DNA metabolic functions: the energy-dependent unwinding of dsDNA to generate the single stranded species utilized as template in these DNA functions. Therefore, a clear understanding of the properties and modes of action of these enzymes would appear very necessary for the elucidation of numerous cellular processes.

The objective of the experimental work presented in this thesis is, therefore, to make some contribution towards the identification and biochemical characterization of the DNA helicases contained in HeLa cells. The results obtained will be embodied in two parts:

- 1)- the properties of a novel DNA unwinding enzyme, human DNA helicase VII (HDH VII) endowed with a very high intrinsic helicase activity and further stimulated nearly a hundred-fold, in a species specific manner, by the replication protein hRPA
- 2)- the functional properties of the separate subunits of HDH II/Ku as well as the effects of phosphorylation, by the DNA-dependent protein kinase (DNA-PK), on the DNA unwinding activity of the recombinant Ku molecule.

2.10. PUBLISHED WORK

- 1). The establishment of the overall purification procedure for the over 15 DNA helicases identified, so far, (see figure 2.7.1) has been the product of many years of work in the Trieste lab. in which I have participated from the beginning and is reported in several papers (refs 116, 166, 167, 170, 171).
- 2). The purification and characterization of HDH VII is totally and exclusively my work and has not been published as yet.
- 3). In the section that deals with the separate subunits of the recombinant HDH II/Ku, the determination of the DNA-dependent ATPase activity of the various forms of Ku was done in collaboration with Dr. Doris Skopac.

This section of my thesis has been published with the title “Functional Properties of the Separate Subunits of Human DNA Helicase II/Ku Autoantigen” in JBC volume 272, no. 47, issue of November 21, 1997, pp 29919-29926.

A reprint of this publication is submitted with the thesis.

3. MATERIALS AND METHODS

3.1. Enzymes, Proteins, Nucleoside Tri-phosphates and Other Reagents

DNA restriction and modifying enzymes and *E.coli* SSB were purchased from New England Biolabs (New England Biolabs Inc. Beverly MA, USA); M13mp18 ssDNA, NTPs, dNTPs, oligonucleotides, and alkaline phosphatase substrate and colour development kit were purchased from Boehringer (Boehringer Mannheim GmbH) as well as DNA Terminal Transferase. The human replication protein hRPA was purified from HeLa cytosol by a modification of the procedure described by Wold et al (179). Heparin-Spharose and DEAE-sephacel resins were purchased from Pharmacia (Pharmacia-LKB Biotech, Uppsala, Sweden). All the protease inhibitors, calf thymus ssDNA-cellulose, dsDNA-cellulose, phospho-cellulose, hydroxyapatite resins, myo-inositol, dephosphorylated β -casein, poly[dI-dC].poly[dI-dC], and goat anti-rabbit antibody were all purchased from Sigma (Sigma Corp. St. Louis MO, USA). [γ - ^{33}P] ATP (5,000 Ci/mmol); [γ - ^{32}P] ATP (3,000 Ci/mmol); [[α - ^{32}P] dATP (3,000 Ci/mmol); [[α - ^{32}P] dCTP (3,000 Ci/mmol)]; [α - ^{32}P] ATP were purchased from Amersham Corp. (Amersham, UK). The cDNA plasmids expressing the recombinant Ku subunits as well as the competent *E.coli* strains were kindly donated by Dr. Simoncsits, A. (ICGEB Trieste).

3.2. DNA Oligonucleotides

Seventeen different oligonucleotides were used to construct the various DNA substrates employed in the characterization of HDH VII. The sequences of these oligonucleotides are as follows:

1). 101mer: 5'-TTG TAA AAC GAC GGC CAG TGA ATT CGA GCT CGG TAC CCG GGG ATC CTC TAG AGT CGA CCT GCA GGC ATG CAA GCT TGG CGT AAT CAT GGT CAT AGG TCT TT-3', used for small linear substrates.

2). 15mer: 5'-CCT CTA GAG TCG ACC-3', complementary to oligonucleotide 1 at nucleotides 45-59, (figure 4.1.8 N).

3). 17mer: 5'-TGG CCG TCG TTT TAC AA-3', complementary to oligonucleotide 1 at nucleotides 1-17, (figure 4.1.8 N).

4). 17mer: 5'-GCA GGT CGA CTC TAG AG-3', complementary to 1 at nucleotides 46-62 (figure 4.1.8 P).

5). 17mer: 5'-GTC GTG ACT GGG AAA AC-3' complementary to oligo. no. 9 and used with its 17mer complementary strand in the substrate in figure 4.1.8 M.

6). 27mer: 5'-ATC CCC GGG TAC CGA GCT CGA ATT CAC-3' complementary to 1 at nucleotides 18-44 (figure 4.1.8 O).

7). 32mer: 5'-CGA CTC TAG AGG ATC CCC GGG TAC CGA GCT CG-3', complementary to M13mp18 DNA at oligonucleotides 6228 to 6259 (Figure 4.1.8 I).

- 8). 33mer: 5'-CTC TAG AGG ATC CCC GGG TAC CGA GCT CGA ATT-3', complementary to M13mp18 DNA at oligonucleotides 6231 to 6263 (Figure 4.1.8 J).
- 9). 47mer: 5'-(T)₁₅ GTT TTC CCA GTC ACG AC (T)₁₅-3'. The central 17 nucleotides are complementary to M13 mp18 at nucleotides 6310-6326 (Figure 4.1.8 A).
- 10). 17mer: 5'-GTT TTC CCA GTC ACG AC-3'. the same as oligonucleotide 5, but without hanging tails (Figure 4.1.8 B).
- 11). 32mer: 5'-(T)₁₅ GTT TTC CCA GTC ACG AC-3'. The same as oligo 5, but with only a 5' hanging tail (Figure 4.1.8 C).
- 12). 32mer 5'-GTT TTC CCA GTC ACG AC (T)₁₅-3'. The same as oligo 5, but with only a 3' hanging tail (Figure 4.1.6 D).
- 13). 54mer: 5'-(T)₅-GAC TCT AGA GGA TCC CCG GGT ACC GAG CTC GAA T-(T)₁₅-3' contains sequences from 6-39 complementary to M13 mp18 ssDNA at nucleotides 6233-6266 (Figure 4.1.8 K, L).
- 14). 50mer: 5'-(A)₁₅-GGT TTT CCC AGT CAC GAC GT-(A)₁₅-3', contains sequences from 16 to 35 complementary to M13mp18 DNA at nucleotides 6308 to 6327 (figure 4.1.8 E).
- 15) 60mer: 5'-(A)₁₅GCC AGG GTT TTC CCA GTC ACG ACG TTG TAA(A)₁₅-3'. Its sequences from 16 to 45 are complementary to M13mp18 at nucleotides 6303 to 6332 (figure 4.1.8 F).
- 16) 70mer: 5'-(A)₁₅GCC AGG GTT TTC CCA GTC ACG ACG TTG TAA AAC GAC GGC C(A)₁₅-3' The sequences from 16 to 55 anneal to M13mp18 at nucleotides 6293 to 6332 (figure 4.1.8 G).
- 17) 42mer: 5'-TCG AGC TCG GTA CCC GGG GAT CCT CTA GAG TCG ACC TGC AGG-3', anneals to M13mp19 ssDNA at nucleotides 6245 to 6286 (figure 4.1.8 H).

3.3. Preparation of The Helicase Substrates

The principal DNA helicase substrate used in the experiments presented in this thesis was the 47mer (oligonucleotide 9 above) annealed to M13 ssDNA, in its central 17 nucleotides, to generate a 3' and 5' double tailed substrate. The structure of this substrate is shown in figure 6 A. About 20 pmol of the oligonucleotide were end-labeled with 20 μ Ci of (γ^{32} P) ATP and 10 units of T4 polynucleotide kinase for 1 hour at 37°C in a 10- μ l reaction mixture containing 70 mM Tris-HCl pH 7.6, 10 mM MgCl₂ and 5 mM DTT. After heat inactivation of the kinase for 3 minutes at 95°C the oligonucleotide was treated with a slight molar excess of M13mp18 ssDNA (~ 4 μ g of DNA), heated for additional 3 min at the same temperature, transferred immediately to 65°C and heated for 10-15 minutes. Annealing of the oligonucleotide to M13 ssDNA was achieved by slowly cooling the reaction mixture down to room temperature in the presence of 20 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 100 mM

NaCl and 1 mM DTT in a final volume of 40- μ l. Non-incorporated radioactivity as well as non-annealed radioactive oligonucleotide were removed by gel filtration over a 5-ml column of Sepharose 4B (Pharmacia biotech.).

For the substrates used to determine the direction of translocation, kinase labelling of the 5' end of the appropriate oligonucleotide (5'-TTG CAT GCC TGC AGG TCG ACT CTA GAG GAT CCC-3') followed exactly the same procedure described above for the preparation of the standard substrate, while labelling at the 3' end was achieved by the addition of one residue of $\alpha^{32}\text{P}$ dATP in a 10- μ l reaction mixture containing 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT and 5 units of DNA polymerase I (Klenow fragment). After annealing to M13 ssDNA was achieved by gradual cooling of the mixture to room temperature, these substrates were separately digested each with 10 units of Hinc II restriction endonuclease in this same buffer to yield linear substrates.

In the case of the linear 5' and 3' double tailed substrate, the 3' end-labelling was carried out in a 50- μ l reaction mixture containing 25 units of Terminal Transferase, 20 μ Ci $\alpha^{32}\text{P}$ dATP, 2.5 mM CoCl_2 , 200 mM potassium cacodylate, 25 mM Tris-HCl pH 6.6 and 250 μ g/ml bovine serum albumin according to the instructions of the supplier. The structures of the various DNA substrates are shown in Figure 4.1.8 A to P.

3.4. Buffers

Unless otherwise specified, all the buffers used in the purification and study of the enzymes presented in this thesis, except buffer G contained 1 mM dithiothreitol, 1 μ M pepstatin, 1 μ M leupeptin, 0.5 mM phenylmethylsulfonyl fluoride and 1 mM sodium metabisulfite. Buffer A contained 20 mM HEPES (pH 7.9 at 4°C), 20% glycerol, 0.2 mM EDTA and 100 mM NaCl. Buffer B contained 20 mM Tris HCl (pH 8.0 or pH 7.5), 5% glycerol, 3 mM MgCl_2 , 0.2 mM EDTA and 100 mM NaCl. Buffer C contained 20 mM HEPES (pH 7.9 at 4°C), 5% glycerol, 3 mM MgCl_2 , 0.2 mM EDTA and 100 mM NaCl. Buffer G contained 6 M guanidinium hydrochloride, 100 mM sodium dihydrogen phosphate, 10 mM Tris-HCl pH 8, and 10 mM β -mercaptoethanol. Buffer R contained 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM MgCl_2 , and 0.2 mM EDTA. The buffer for the gel filtration chromatography was the same as buffer C but contained 2% glycerol.

3.5. Extraction of Proteins from HeLa Cell Nuclei

HeLa nuclear proteins were extracted from frozen, *in vitro* cultured cells according to a protocol originally described by Dignam, J. et al. (180), as follows: All the steps in the preparation of the nuclear extract were performed at 4°C. 150 g of HeLa cells were suspended in 800 ml (five cell-pellet volumes) of buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10mM KCl and 0.5 mM DTT, and allowed to stand on ice for 10 minutes. The cells were collected by centrifugation for 10 minutes at 2000 r.p.m. in a Sorvall HG4L rotor, re-suspended in 320 ml (two initial cell-pellet volumes) of the same buffer and lysed with 20 strokes of a Kontes Dounce all-glass homogeneizer (B-type tight-fitting pestle). The homogenate was centrifuged as before to pellet the nuclei, and the supernatant was carefully decanted and set aside for the preparation of the single stranded DNA binding protein, RPA. The loose pellet of nuclei from this low speed centrifugation was subjected to a second centrifugation for 20 minutes at 25,000 g in a Sorvall super speed ss34 rotor to remove residual cytoplasmic material. These crude nuclei were suspended in 230 ml (3 ml/10⁹ cells) of buffer containing 20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT with the Dounce A-type (loose-fitting) pestle. The resulting suspension was stirred gently for 30 minutes with a magnetic stirring bar, centrifuged for 30 minutes at 25,000 g in a Sorvall ss34 rotor, and the pellet was discarded. The supernatant, 215 ml of nuclear extract, was precipitated with 75.2 g of ammonium sulphate (0.35 g/ml), re-suspended and dialyzed extensively against buffer containing 20 mM HEPES (pH 7.9), 20% glycerol, 100 mM NaCl and 0.2 mM EDTA. A total of 1240 mg of HeLa nuclear proteins were extracted in this procedure and were used in the purification of HDH VII.

3.6. Purification of hRPA from HeLa Cytosol

Replication protein A (RPA) was purified from HeLa cytosol by a modification a protocol described by Wold, M.S. et al.(179), by column chromatography through the following resins:

- a). phosphocellulose
- b). hydroxyapatite
- c). DEAE-sephacel
- d). ssDNA-cellulose

The buffer used in this purification contained 20 mM HEPES (pH 7.9 at 4° C), 0.25% myo-inositol and 100 mM KCl. RPA activity was monitored, throughout the purification procedure, by electrophoretic gel mobility shift assay (EMSA) using a ³²P end-labeled 25mer oligonucleotide as ssDNA probe. The sequence of this DNA probe was the following: 5'GATCTCGCATCACGTGACGAAGATC-3'.

The supernatant from the low speed centrifugation (300 ml), in the nuclear extract preparation described above, were treated with 33 ml (0.11 volume) of buffer containing 300 mM HEPES (pH 7.9), 1.4 M KCl and 30 mM MgCl₂, and centrifuged for 60 minutes at 100,000 g in Beckman swing-out sw 42 rotor. The resulting supernatant, designated the S100 fraction, was dialyzed against 2 lt of purification buffer with three changes of this buffer. After centrifugation for 30 minutes at 25,000 g in Sorvall ss34 rotor, the supernatant was used to isolate hRPA while the pellet was discarded.

About 900 mg of cytosolic proteins were loaded onto a phosphocellulose column (2.6 cm diameter x 25 cm) pre-equilibrated in the purification buffer. RPA eluted in the flow through fractions in a total of 455 mg of proteins which were immediately loaded onto a 50-ml hydroxyapatite column. After extensive washing of this column to remove unbound proteins, RPA was eluted with the purification buffer containing 100 mM phosphates (pH 8). A total of 150 mg of proteins were pooled in this elution and dialyzed against buffer containing 0.01% NP40 to remove the phosphates. The dialyzed protein solution was clarified by centrifugation and loaded onto a 20-ml DEAE-sephacel column. This column was washed and bound proteins were eluted with 300 ml of a linear gradient (15 column volumes) from 100 mM to 300 mM KCl. RPA eluted between 150 mM and 220 mM KCl, and a total of 24.5 mg of proteins were pooled. This pool was diluted to adjust the salt concentration to 100 mM and loaded onto an 8-ml ssDNA-cellulose column. After washing, the column was eluted with 200 ml (25 column volumes) of a linear gradient from 100 mM to 1.0 M KCl, and about 0.2 mg of homogeneous hRPA eluted around 300 mM KCl. This protein pool was frozen in small aliquots and used directly in studies of stimulation of helicase activity by the single strand binding protein.

3.7. Electrophoretic Mobility Shift Assay.

DNA gel retardation experiments were performed on a ³²P 5' end-labeled 25mer oligonucleotide 5'-GATCTCGCATCACGTGACGAAGATC-3' (or duplex 25mer when annealed to its complementary sequence as appropriate), as follows: the protein samples assayed were made to react with the DNA probe in a 20-μl reaction mixture containing 20 mM Tris-HCl (pH 8), 1 mM MgCl₂, 4 mM ATP, 60 mM KCl, 8 mM DTT, 4% (w/v) sucrose, 80 μg/ml bovine serum albumin and ~0.05 pmol ³²P labeled DNA probe (generally

10,000-30,000 cpm). This mixture was incubated for 30 minutes at room temperature, and the products were subsequently separated on a 5% non-denaturing PAGE in buffer containing 45 mM Tris, 45 mM boric acid and 1 mM EDTA at 4°C. After this electrophoresis, the gel was dried and bands were visualized by autoradiography and quantified by means of the Packard Electronic Autoradiography Instant Imager. 1 unit of DNA binding activity is defined as the amount of protein that retards 1 fmol of DNA probe in a standard gel retardation assay.

3.8. Helicase and ATPase Assays

The helicase assay measured the displacement of an end-labeled oligonucleotide from a partial duplex DNA molecule. The principal DNA substrate used in the helicase assays in this study contained a ^{32}P 5' end-labelled 47mer oligonucleotide annealed to M13 ssDNA in its central 17 nucleotides, yielding a partial duplex with 5' and 3' hanging tails of 15 nucleotides each. The sequence of this oligonucleotide as well as the structure of the substrate are shown in figure 4.1.6 (panels A to P). The 10- μl reaction mixture contained 20 mM Tris HCl (pH 8.0), 3 mM MgCl_2 , 8 mM ATP, 60 mM KCl, 8 mM DTT, 4% (w/v) sucrose, 80 $\mu\text{g/ml}$ BSA, about 0.005 pmol of ^{32}P labeled substrate (generally ~ 5000 cpm) and the enzyme fractions. After incubation for 30 minutes at 37°C, the reactions were stopped by the addition of 10 mM EDTA, 5% glycerol, 0.3% SDS and 0.03% bromophenol blue, and the products were separated on a 12% non-denaturing PAGE at room temperature in 90 mM Tris, 90 mM boric acid and 2 mM EDTA. The gel was dried and DNA unwinding was visualized by autoradiography, and quantitated by means of the Packard Electronic Autoradiography Instant Imager (Packard Instruments Inc., Downers Grove, IL 60515, USA).

The ATPase assays contained 20 mM Tris HCl (pH 8.5), 1 mM MgCl_2 , 1 mM ATP, 30 mM KCl, 8 mM DTT, 4% (w/v) sucrose, 80 $\mu\text{g/ml}$ BSA, 5 μCi γ - ^{32}P ATP, 1 μg ssDNA and the enzyme fractions. Assays were performed in the presence or absence of ssDNA and ATP hydrolysis at 0 min was monitored as control. The mixture was incubated for 30 minutes at 37°C and stopped by chilling to 4°C. 1- μl of the mixture was spotted on polyethyleneimine-cellulose thin-layer strip and ascending chromatography was performed in 0.5 M LiCl, 1 M formic acid for 15 minutes at room temperature. The strip was dried and ATPase activity was revealed by autoradiography and quantitation was also carried out with the Packard Instant Imager.

3.9. Estimation of Protein Molecular Weight by Glycerol Gradient Sedimentation and Gel Filtration Chromatography

The determination of the molecular weight for HDH VII by a combination of these two methods was carried out as originally described by Siegel and Monty (181). Using a HSI Auto Densi-Flow II C (Buchler Instruments, Lenexa, KS), several 4 ml tube linear gradients (15%-35%) were prepared in buffer C for the glycerol gradient centrifugation. 200- μ l (~1.4 μ g) of pure HDH VII were carefully layered on the top of the gradient and, in the same manner, various mixtures of selected molecular weight markers were layered on the other gradient-containing tubes. These gradients were centrifuged for twenty hours at 4°C at 55,000 rpm in Beckmann swing out SW 65 rotor. Fractions of 330- μ l were collected from the top of each tube, using the same Auto Densi-Flow gradient maker/fractionator at the end of the centrifugation. Fractions from the HDH VII-containing tube were assayed for helicase activity whereas fractions from the tubes of the molecular markers were subjected to SDS-PAGE analysis to reveal their pattern of separation.

For the gel filtration, I used a Superdex 75TM HR 10/30 (Pharmacia-LKB Biotech, Uppsala, Sweden) column on the Fast Protein Liquid Chromatograph (FPLC, Pharmacia). After an adequate equilibration of the column, this was appropriately calibrated by several runs of various mixtures of selected molecular weight standards. Subsequently, 100- μ l of concentrated HDH VII (~4 μ g) were injected onto the column and 1-ml fractions were collected and assayed for DNA unwinding activity. These fractions were then concentrated, as before, by ultra-filtration to about 50- μ l each and analyzed on SDS-10% polyacrylamide gel electrophoresis for the presence of protein bands.

3.10. Affinity Labeling with ATP

The affinity labeling of HDH VII with α ³²P ATP was carried out as follows: a 20- μ l reaction mixture containing 20 mM Tris-HCl (pH 8), 3 mM MgCl₂, 60 mM KCl, 1 μ Ci of α ³²P ATP and 35 ng of HDH VII was incubated at 37°C for 1 minute and later, embedded in ice, was exposed to uv irradiation at 254 nm for 10 minutes in the dark. In parallel, a negative control which contained all the components of the reaction mixture was prepared but was not uv-irradiated. This control was necessary to reveal effective binding of ATP to the helicase and the formation of covalent bonding between the reactants upon uv irradiation. After this treatment, both samples, Uv-treated and non-treated, were electrophoresed on SDS-10% PAGE according to Laemli (182). The gel was washed for 18 hours with 10% acetic acid to remove non-bound radioactivity, dried and exposed for autoradiography.

3.11. Western Blot Analysis of Protein Fractions

Proteins were separated by SDS-10% polyacrylamide gel electrophoresis according to Laemli (1982), and electro-transferred to nitrocellulose membrane. The membrane was saturated with albumin by incubation in buffer containing 10 mM Tris-HCl pH 8.5, 150 mM NaCl, 0.2% Tween 20 and 5% low-fat milk for one hour at 37°C. The buffer was discarded and replaced with 10-ml of fresh aliquot containing a 1000-fold (final) dilution of the appropriate primary rabbit polyclonal antibody and incubation was carried on at room temperature for two hours. The solution of antibody was removed and the membrane was washed three times for 10 minutes each with the same buffer to remove non-reacted excess antibody. Subsequently, the membrane was treated with 10 ml of buffer containing a 2500-fold final dilution of goat anti-rabbit alkaline phosphatase-conjugate second antibody (Sigma, St. Louis MO) and incubated for another one hour at room temperature. The membrane was then washed, in succession, once with 10 ml of the same buffer without the antibody, once with buffer without low-fat milk, and twice with buffer containing 10 mM Tris-HCl and 150 mM NaCl. Immunoreactivity was revealed by incubating the membrane for a few minutes with an aqueous solution of the alkaline substrate and colour development reagent.

3.12. Expression and Purification of Recombinant HDH II/Ku.

E. coli cells (strain BL 21 (DE3)(pLysS)) were transformed with the respective plasmids for the 72 kDa or the 87 kDa subunits of HDH II/Ku and grown in LB medium (1983) containing 75 mg/L ampicillin and 25 mg/L chloramphenicol with vigorous shaking to an optical density of 0.5 at 600 nm. Protein expression was induced by addition of IPTG to a final concentration of 0.4 mM and the cultures were shaken for a further 2.5 hours. Cells were harvested by centrifugation and re-suspended in lysis buffer (50 mM Tris HCl, pH 8, 2 mM EDTA and protease inhibitors). After two freeze/thaw cycles, the extremely viscous solution was fluidified by sonication (the tubes were constantly cooled on ice) and centrifuged. The supernatants were carefully decanted from the loose pellets, and these latter were suspended and washed once in detergent solution (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.2 M NaCl, 1% NP40 and 1% sodium deoxy-cholate), and centrifuged as before. The pellets were suspended in washing buffer (0.5% Triton X 100, 2 mM EDTA and 0.5 mM PMSF), and washed about five times until a compact pellet of inclusion bodies was obtained for each Ku subunit.

These pelleted inclusion bodies were solubilized in buffer G (denaturing buffer) and purified by gel filtration on Sephacryl S300 (Pharmacia, Uppsala, Sweden) at 25°C in the same buffer to eliminate possible low molecular weight contaminants of bacterial origin. After SDS-PAGE analysis, the very clean fractions from this chromatography were pooled for each subunit and carried over for refolding both as the separate subunits as well as in an equimolar mixture to reconstitute the recombinant heterodimer.

3.13. Refolding and Reconstitution of Enzymatic Activity

The separate subunits of HDH II/Ku were treated individually with a solution of a non-detergent solubilizing and stabilizing agent NDBS 195 (dimethyl ethylammonium propane sulfonate) at a final concentration of 0.2 M. After this treatment, the separate subunits as well as an equimolar mixture of these were successfully refolded by dialysis at 4°C in buffer R. An earlier attempt at refolding with a 0.1 M final concentration of this solubilizing agent had resulted in slight precipitation of the separate subunits during dialysis. The refolded samples (separate subunits and the heterodimer) were centrifuged at 10 000 rpm at 4°C in a Sorval ss 34 rotor for 20 minutes and the supernatants were used either directly or after one further purification step, for DNA binding, helicase and ATPase assays.

3.14. dsDNA-Sepharose Affinity and Mono Q Anion Exchange Chromatography

The dsDNA affinity resin used for the purification of the recombinant HDHII/Ku heterodimer was prepared as described by Csordas Toth et al (165) except that the oligonucleotides used were the following: 5'-GATCTCGCATCACGTGACGAAGATC-3' and its complementary sequence both synthesized chemically at ICGEB on an Applied Biosystems DNA synthesizer. Annealing of these two sequences generated a 25 bp blunt-end oligonucleotide which was directly coupled to cyanogen bromide-activated Sepharose (Pharmacia) after phosphorylation with T4 polynucleotide kinase as described (Kadonega and Tijan 1986) (184). This matrix was packed in a 15 ml column and equilibrated in buffer C for the affinity purification of the enzyme. Refolded recombinant HDH II/Ku heterodimer was adjusted to the salt molarity of buffer C and loaded onto the dsDNA-Sepharose column. After washing to remove unbound material, bound proteins were eluted with 10 column

volumes of a linear gradient from 0.1M to 1M NaCl. DNA binding and helicase activities co-eluted at ~ 0.25 M NaCl.

Mono Q HR5/5 (1ml) column was equilibrated on FPLC (Pharmacia) in buffer C. The refolded separate subunits were adjusted to the composition of this buffer and applied separately onto the Mono Q column. After extensive washing of the column, bound material was eluted in each case with 20 column volumes of 0.1 M-1.0 M NaCl linear gradient. Purification was followed by monitoring the DNA unwinding activity in the various fractions for the 72kDa subunit, by DNA binding and helicase assays for the recombinant heterodimer, whereas for the 87 kDa subunit, which showed no helicase activity, purification was followed by SDS-PAGE analysis of the fractions.

3.15. Purification of the DNA-Dependent Protein Kinase From HeLa Nuclei

The DNA-dependent protein kinase (DNA-PK) was purified by chromatography, after ammonium sulphate precipitation of the nuclear extract, through the following resins:

- a) Bio rex 70
- b) Mono Q
- c) dsDNA-Sepharose

The general scheme for this purification is shown in figure 2.7.1.

3.16. DNA-Dependent Protein Kinase Assay

The DNA dependent protein kinase assay measures the transfer of the γ -phosphate from a donor nucleoside-triphosphate, in the presence of DNA, to a chosen substrate. The substrate used to characterize the kinase activity in this study was dephosphorylated β -casein, and assays were run in the presence or absence of DNA. The 10- μ l reaction mixture contained 20 mM HEPES pH 7.9, 3 mM $MgCl_2$, 1 mM DTT, 50 mM KCl, 100 μ M ATP containing [γ ^{32}P ATP], and 0.8 μ g dephosphorylated casein. Assays were started by adding 1 μ l of the kinase sample and the reaction mixtures were incubated for 20 minutes at 37 $^{\circ}$ C. Kinase reactions were terminated by adding SDS-PAGE protein denaturing buffer and the products were separated electrophoretically on a 10% acrylamide gel. The gels were dried and protein kinase activity was revealed by autoradiography, and the DNA-dependent kinase activity was calculated as the incorporation of γ ^{32}P ATP in the presence of DNA minus the incorporation in the absence of DNA.

4. RESULTS

Part I - HDH VII

4.1. HeLa DNA Helicase VII is a Novel Human Protein: Its DNA Unwinding Activity is Specifically Stimulated by the Single-Stranded DNA Binding Protein RPA

Human DNA helicase VII (HDH VII) was purified to homogeneity in the course of a systematic study of the helicases contained in HeLa cell nuclei. This enzyme possesses the highest specific activity amongst all the helicases so far characterized in this laboratory, and this activity is further stimulated in a species-specific manner by RPA.

4.1.1. Purification of HDH VII

HeLa nuclear extract was prepared as described in materials and methods. From 150 g of *in-vitro* cultured cells a total of 1.6 g of nuclear proteins were extracted, precipitated with ammonium sulphate (0.35 g/ml), re-suspended and dialyzed in buffer A. A total of 1240 mg of proteins were recovered and were loaded onto a Bio Rex 70 weak cation exchange column (2.6 cm diameter x 25 cm), (Bio Rad Lab., Richmond USA) pre-equilibrated in buffer A. The flow through fractions which contained DNA unwinding activity were pooled and used to isolate other helicases as described elsewhere (168). The column was washed with several volumes of buffer A and subsequently eluted with a six-column volume linear gradient from 0.1 M to 0.6 M NaCl. The DNA unwinding activity which eluted between 0.25 M and 0.4 M NaCl was pooled whereas a second peak of activity which eluted during a successive gradient from 0.6 M-1.5 M NaCl contained other DNA unwinding activities already described (167, 169).

The activity eluting in the first gradient was concentrated by ammonium sulfate precipitation (0.35g/ml), dialyzed in buffer B and loaded onto an 8-ml FPLC strong anion exchange column (Mono Q HR 10/10, Pharmacia-LKB Biotech, Uppsala, Sweden) equilibrated in the same buffer. HDH VII eluted in the flow-through fractions, whereas the protein fractions that eluted from this column, in the subsequent gradient, contained other helicases described elsewhere (116, 170), as well as the DNA-dependent protein kinase (DNA-PK) which was later purified and used for the study of the effect of phosphorylation on the helicase activity of HDH II/Ku. Up to this stage of the purification, it was not possible to precisely quantitate the activity of HDH VII due to the presence of other helicases as well as contaminating nucleases (Table 4.1.1). The flow-through fractions were pooled, dialyzed in

buffer C and fractionated on a 1-ml FPLC strong cation exchange column (MonoS HR 5/5, Pharmacia). The predominant DNA unwinding activity which eluted between 0.2 M and 0.3 M NaCl contained HDH VII as well as a helicase of the RecQL family (185). These two activities were successfully separated by passage through a 5-ml Heparin Sepharose column (Pharmacia-LKB) from which HDH VII eluted between 0.3 M and 0.45 M while RecQL eluted earlier. The homogenous enzyme - a total of 110 µg - was isolated by a subsequent chromatography on a 1-ml ssDNA-cellulose column (Sigma Co., St. Louis MO USA)

Table 4.1.I summarizes the purification of HDH VII while table 4.1.II shows the DNA unwinding observed for the enzyme under different assay conditions. Figure 4.1.1. panel A shows the protein elution profiles of the different purification steps while panel B shows the SDS-PAGE analysis of the pooled fractions from these purification steps. Figure 4.1.2 panels A and B show the DNA helicase activity profile and the corresponding SDS-PAGE analysis of some of the active fractions of the final column. One main protein band is visible corresponding to a Mr of 32 kDa, plus a less intense one with a slightly higher Mr (see below for a comparison with the Mr of the native molecule). Figure 4.1.2 panel C shows uv crosslinking with α ³²P ATP.

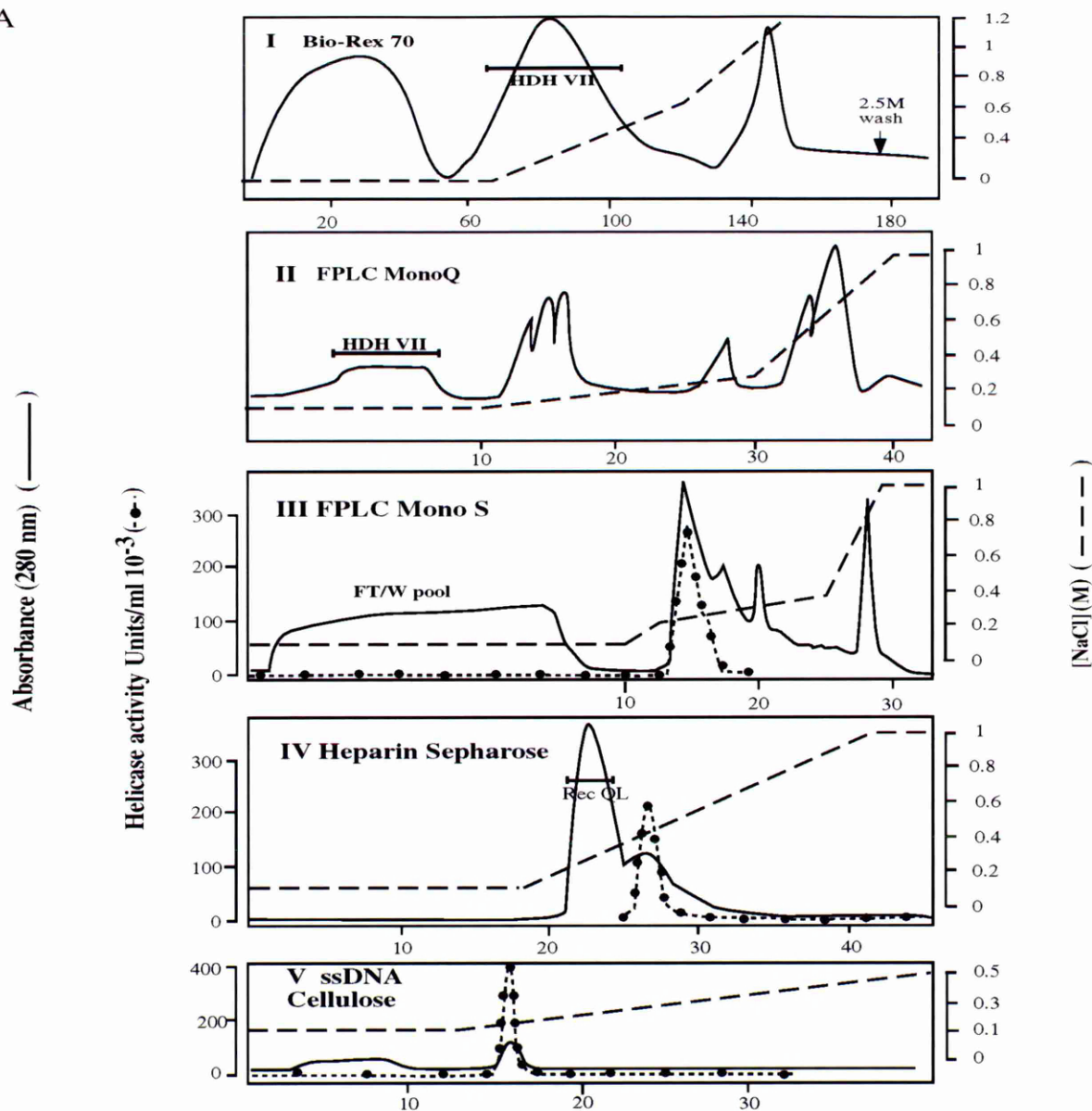
Table 4.1.I. Purification of Human DNA Helixase VII

Fraction	Volume (ml)	Total Proteins(mg)	Tot.Helicase Units*	Sp. Activity (U/mg)
I	200	1,240	N.D.**	N.D.**
II	320	224	N.D.**	N.D.**
III	65	31.8	1.04 x 10⁷	3.2 x 10⁵
IV	19	9.5	7.6 x 10⁶	8 x 10⁵
V	18	0.9	2.6 x 10⁶	3 x 10⁶
VI	16	0.110	2.1 x 10⁶	1.9 x 10⁷

*One unit of helicase activity is the amount of enzyme that unwinds 1% of the substrate in one minute at 37 C in the linear range of enzyme concentration dependence.

** Not determined

A



B

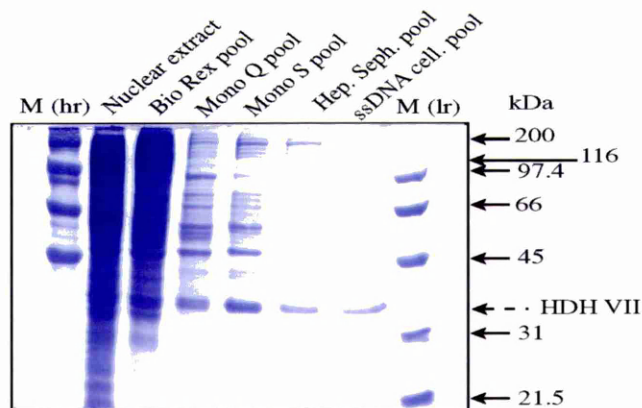


Figure 4.1.1. **Purification of HDH VII.** Panel A: protein elution and helicase activity profiles (where quantitated) from Bio Rex 70 (I), Mono Q (II), Mono S (III), Heparin Sepharose (IV), and ssDNA-cellulose (V) steps. The molar range of salt is indicated in each case. Panel B, SDS-PAGE analysis of the pooled fractions. Lane 1, high-range markers; lane 2, HeLa nuclear extract (70 μ g); lane 3, Bio Rex pool (30 μ g); lane 4, Mono Q pool (15 μ g); lane 5, Mono S pool (12 μ g); lane 6, Heparin Sepharose pool (5 μ g); lane 7, homogeneous HDH VII (0.6 μ g); lane 8, low-range markers. Proteins were stained with Coomassie Brilliant Blue. The masses of the markers are indicated on the right hand margin and the position of pure HDH VII is also shown.

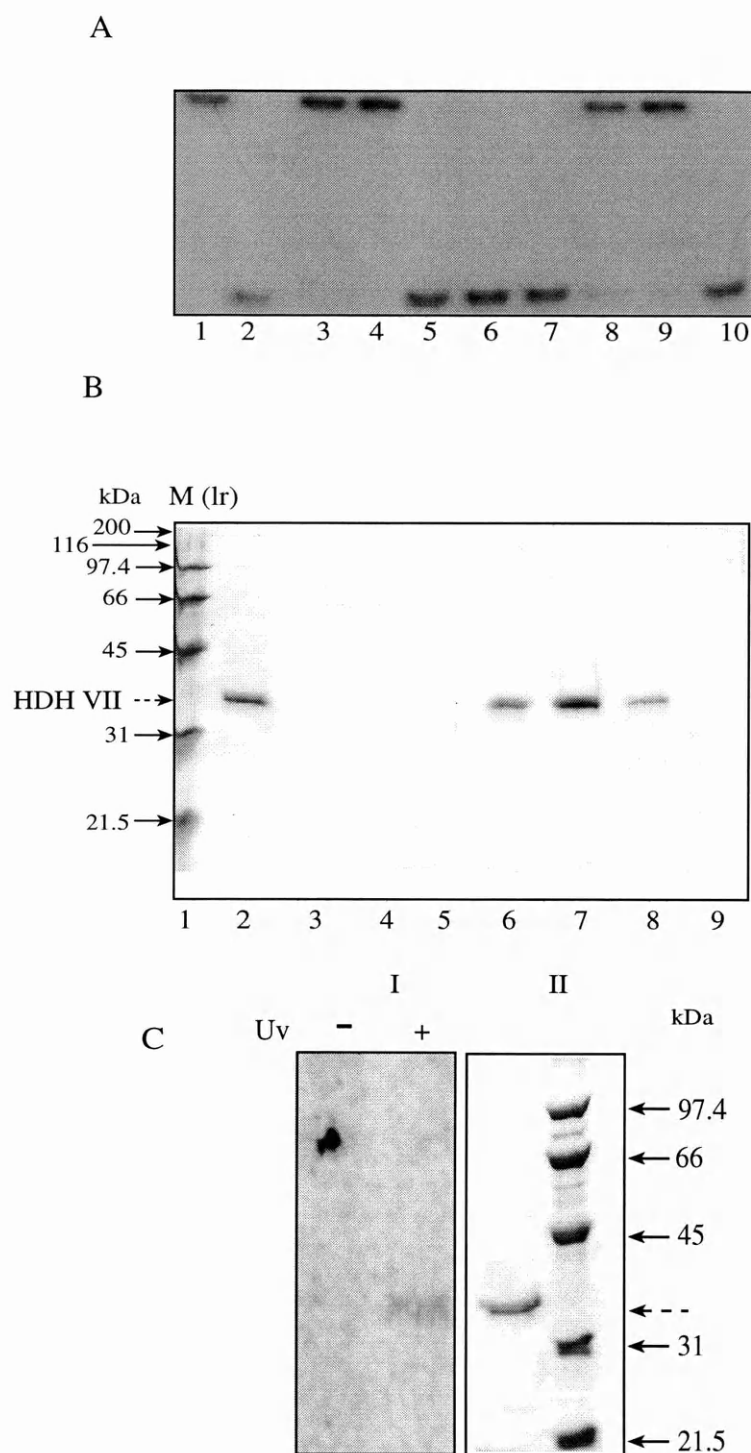


Figure 4.1.2. **Protein Elution and Helicase Activity Profile of the Last Purification Step.** Panel A, helicase assay: lane 1, substrate alone; lane 2, activity loaded on the column; lanes 3-9, FT/wash pool, fractions 14, 15, 16, 17, 18 and 19 respectively; lane 10, heat denatured substrate. 1- μ l aliquots of these fractions were assayed. Panel B, SDS-PAGE analysis: lane 1, low-range markers; lanes 2-9, protein loaded on the column, FT/wash pool, fractions 13, 14, 15, 16, 17 and 18 respectively. 25- μ l of each fraction were analyzed. Panel C, uv crosslinking with ATP. Sample irradiated with uv is indicated (+) while non-irradiated sample is marked (-). A slice of the corresponding coomassie-stained SDS-PAGE is included to reveal the mass of the radioactive protein bands.

4.1.2. General Properties of the Enzyme

Purified HDH VII retained over 50% of its activity when stored for several weeks at 4°C and was completely stable for at least 6 months when stored in 50% glycerol at -20°C. However the unwinding activity is very sensitive to freezing and over 50% of this activity is lost in one single freeze/thaw cycle. HDH VII is inactive on RNA-RNA substrates and on DNA-RNA hybrids (data not shown). Affinity labelling with (α ^{32}P) ATP, illustrated in figure 4.1.2 panel C, shows a diffuse radioactive band in the region of 32-kDa indicating that both of the closely migrating bands which constitute this enzyme bind ATP independently.

Table 4.1.II. Reaction Requirements of Human DNA Helicase VII

Assay conditions	% of unwinding
Complete	> 95
- ATP	< 2
- ATP + dATP	> 90
- ATP + CTP (8 mM) or dCTP (8 mM)	< 2
- ATP + GTP (8 mM) or dGTP (8 mM)	< 2
- ATP + dTTP (8 mM) or UTP (8 mM)	< 2
-ATP +ADP (8 mM) or AMP (8 mM)	2%
- MgCl ₂	< 2
- MgCl ₂ +CaCl ₂ (ZnSo ₄ , MnCl ₂ , CdCl ₂ , CoCl ₂ , NiCl ₂ , CuCl ₂)	< 2
+ KCl or NaCl (250 mM)	< 2

4.1.3. Time Course of DNA Unwinding by HDH VII

The percentage of DNA substrate unwound by a given amount of HDH VII was measured as a function of the reaction time. The results were quantitated and reported in figure 4.1.3 panels A and B. DNA unwinding by 0.15 ng of HDH VII appeared a rapid process since about 50% of the substrate was displaced within five minutes and the unwinding reaction seemed complete in ten minutes (panel A). However, this reaction showed a linear and progressive dependence on the reaction time when 0.05 ng of enzyme were used

(panel B) and about 100% of the DNA substrate used in the assay was unwound in 30 minutes.

4.1.4. Dependence of DNA unwinding on ATP, Mg^{2+}

DNA unwinding was monitored in the presence or absence of ATP and of magnesium chloride. The results which are shown in figure 4.1.4 clearly indicate that DNA unwinding by HDH VII is totally dependent on ATP and magnesium.

4.1.5. Dependence of DNA unwinding on the concentration of ATP, Mg^{2+} and KCl, and Determination of K_m for ATP

The results of a quantitative analysis of the helicase activity of HDH VII in the presence of increasing concentrations of ATP, Mg^{2+} and KCl are shown in graph form in figure 4.1.5 panels A, B and C respectively (inset in each panel is the autoradiogram of the relative helicase activity gel). HDH VII exhibits appreciable DNA unwinding activity in a wide range of ATP concentrations with an optimal concentration of 8mM of this co-factor. The highest tested ATP concentration of 20 mM did not inhibit the activity of the enzyme (panel A). Only dATP could efficiently substitute ATP in the unwinding reaction while all the other canonical NTPs and dNTPs could not sustain any appreciable DNA unwinding (table 4.1.II). The DNA unwinding activity of HDH VII increases with increasing Mg^{2+} concentration, reaches a peak at 3 mM $MgCl_2$ and drops off sharply at a final concentration of 5 mM for this cation (panel B). None of the tested common cations could substitute Mg^{2+} in the DNA unwinding reaction, indicating a specific requirement for this cation by the helicase (table 4.1.II). Salt concentrations up to 20 mM do not have any significant inhibitory effect on the DNA unwinding activity of HDH VII but a final concentration of 250 mM NaCl or KCl completely abolishes the enzymatic activity (panel C).

Evaluation of the DNA unwinding in the presence of increasing concentrations of ATP gave an estimated K_m value of 5.5 mM for this co-factor (figure 4.1.5).

4.1.6. Dependence of DNA Unwinding on Enzyme Concentration

The DNA helicase activity of HDH VII was assayed in the presence of serial dilutions of the enzyme, and the observed DNA unwinding was quantitated. Figure 4.1.6. panels A and B show these results as the autoradiogram of the helicase assay (panel A), and its graph form (panel B). The concentration dependence of DNA unwinding by HDH VII follows a sigmoidal type of curve typical of enzymes with a cooperative type of binding.

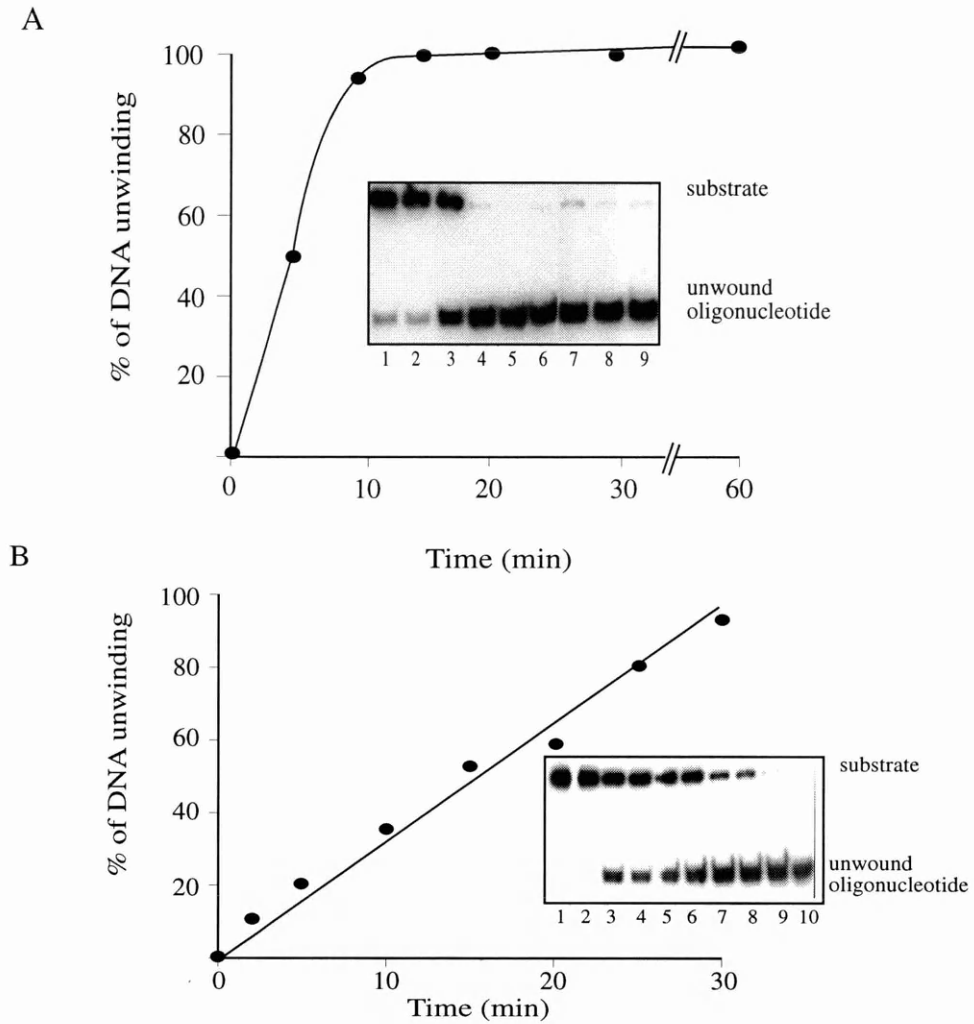


Figure 4.1.3 Time Course of DNA unwinding by HDH VII. Panel A, assay with 0.15ng of enzyme: lane 1, substrate alone; lanes 2-9, substrate with 0.15ng of enzyme incubated for 0 min, 5 min, 10 min, 15 min, 20 min, 30 min and 60 min respectively. Panel B, assay with 0.05ng of enzyme: lane 1, substrate alone; lanes 2-9, substrate with 0.05ng of enzyme incubated for 0 min, 2 min, 5 min, 10 min, 15 min, 20 min, 25 min and 30 min respectively; lane 10, heat denatured substrate

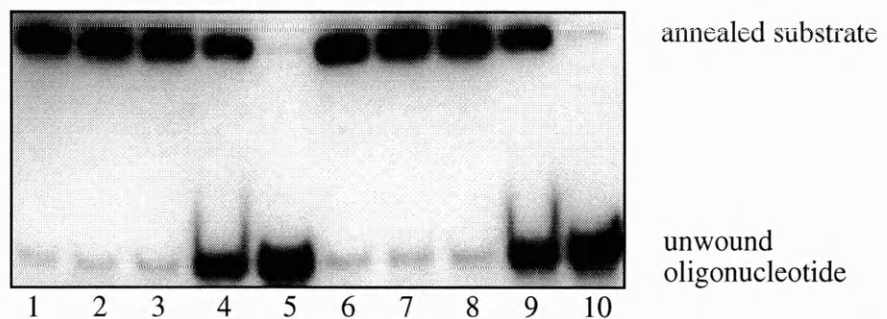


Figure 4.1.4. Dependence of helicase activity on ATP and $MgCl_2$: helicase assay was run with 0.05ng of HDH VII in the presence or absence of ATP and $MgCl_2$. Lane 1, substrate with lane 2, substrate with enzyme, without ATP or $MgCl_2$; lane 3 substrate with ATP and $MgCl_2$ but without enzyme; lane 4, substrate with enzyme, ATP and $MgCl_2$; lane 6, substrate with ATP, without enzyme and $MgCl_2$; lane 7, substrate with enzyme and ATP but without $MgCl_2$; lane 8, substrate with ATP and $MgCl_2$ but without enzyme; lane 9, substrate with enzyme, ATP and $MgCl_2$; lanes 5 and 10, heat denatured substrate.

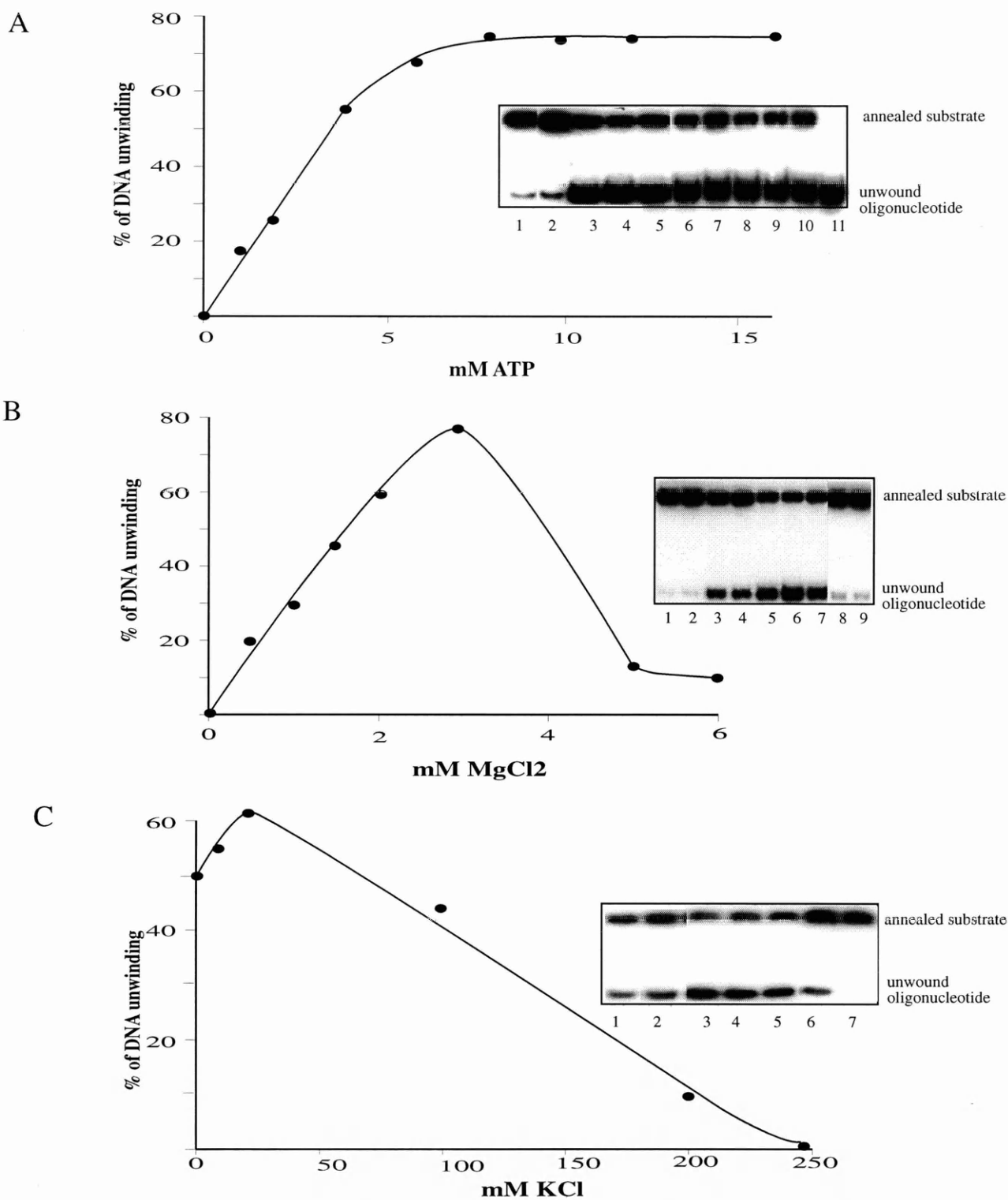


Figure 4.1.5 Dependence of Helicase Activity on Concentrations of ATP, MgCl₂ and KCl: helicase assays under varying conditions; Insets are the relative autoradiograms; Panel A, ATP curve: lane 1, substrate with 8mM ATP alone; lane 2, substrate with 0.1 ng of HDH VII without ATP; lanes 3-10, substrate with 0.1 ng of enzyme and 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6mM, 8mM, 10 mM, 15 mM and 20 mM ATP respectively; lane 11, heat denatured substrate. Panel B, MgCl₂ curve: lane 1, substrate with 3 mM MgCl alone, lanes 2-9, substrate with 0.1 ng of HDh VII and 0.0 mM, 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, 3.0 mM, 5.0 mM and 6.0 mM MgCl₂ respectively. Panel C, KCl curve: lane 1, substrate with 50 mM KCl alone, lanes 2-7, substrate with 0.1 ng of HDH VII and 0 mM, 10 mM, 20 mM, 100 mM, 200 mM, 250 mM KCl respectively.

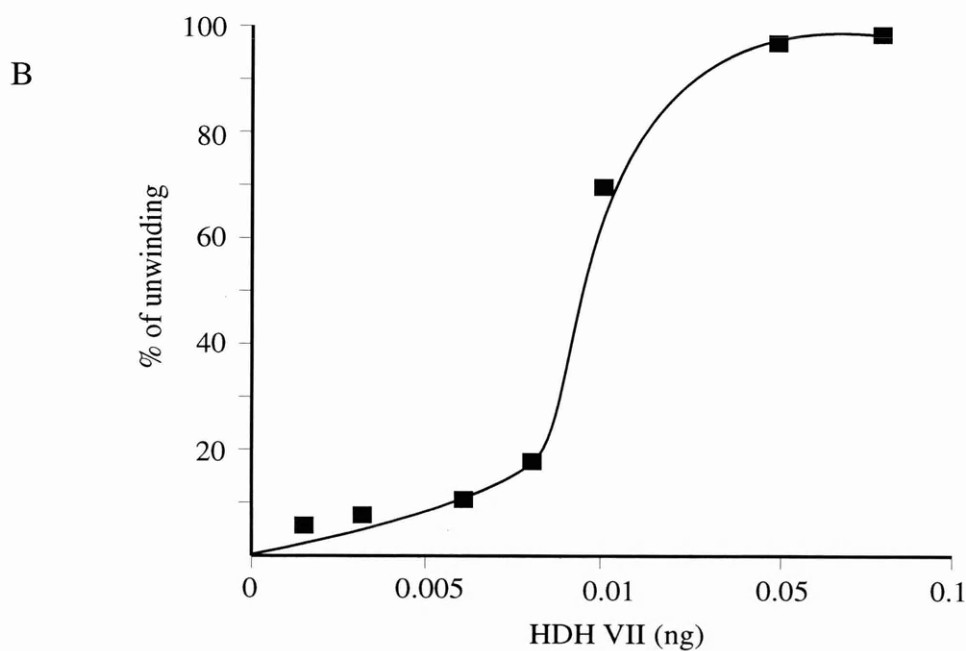
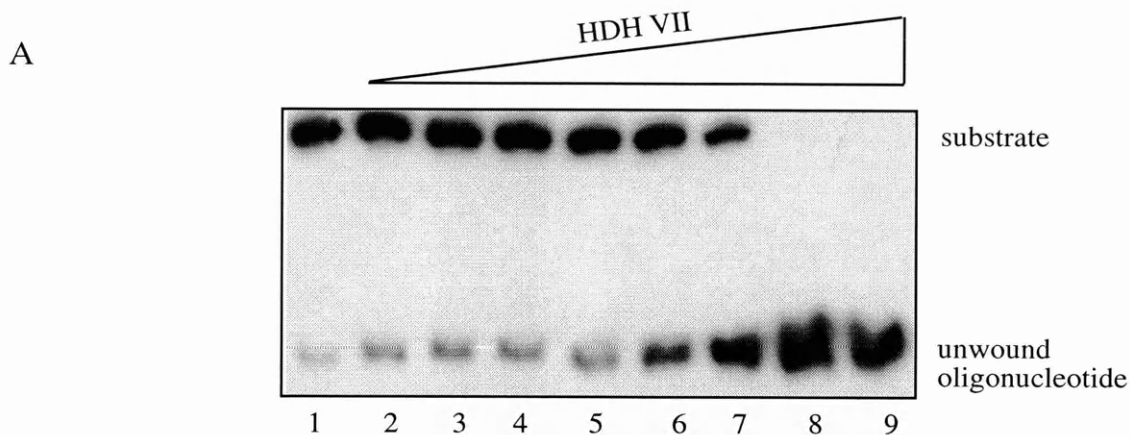


Figure 4.1.6. **Dependence of Helicase Activity on Enzyme Concentration.** Pure HDH VII was diluted and tested for DNA unwinding. Panel A: Lane 1, substrate alone; lanes 2-9, substrate with approximately 0.001ng, 0.003ng, 0.006ng, 0.008ng, 0.01ng, 0.05ng and, 0.1ng of HDH VII respectively. Panel B: graph representation of the data in panel A.

4.1.7. HDH VII Exhibits Double Polarity of Translocation

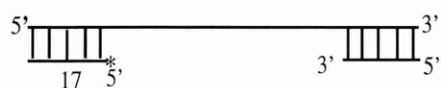
In order to determine the polarity of unwinding by HDH VII, I measured its activity on the two substrates shown in Figure 4.1.7. DNA unwinding is observed with the same efficiency both on the substrate designed to detect a 5' to 3' movement (panel A) and on the opposite one (panel B) designed to detect a 3' to 5' polarity. Since, as will be shown below, HDH VII does not unwind blunt-end duplexes, it follows that the enzyme moves in both directions along the bound DNA strand.

4.1.8. Activity of HDH VII with Different Substrates

Figure 4.1.8, panel A shows the activity of HDH VII with the standard substrate used for the purification, namely an M13 single-stranded circular DNA bound to an end-labelled 47mer oligonucleotide annealed to it by its 17mer central portion, thus bearing two 15 bases of unannealed tails at its 5' and 3' ends. 0.15 ng of HDH VII, sufficient to unwind 90% of this substrate, shows only a minor reduction in efficiency when either or both tails are removed (see panels B to D).

The high DNA unwinding rate of HDH VII encouraged me to investigate its activity in the presence of substrates having double-stranded portions of different length. Surprisingly, as shown in panels E to H, the unwinding activity is hardly detectable on substrates with an annealed portion of 20 or more base pairs (panels E-J). Even the addition of only one or two nucleotides to the 17 bp annealed portion, reduces the unwinding ability to barely detectable rates on linearized substrates, similar to the ones used for the polarity studies (see panels I and J). Linear substrates, with a 5' or 3' tail hanging to the 17 bp duplex (see panels K and L), are unwound at about half the rate as on the circular ones, an observation consistent with the double polarity of movement of HDH VII, since with the latter substrates the enzyme can act at the same time on both sides of the duplex, whereas, as shown by the results reported in panel M, blunt-ended substrates cannot be unwound by the enzyme. Furthermore, as shown in panel O, the enzyme cannot functionally bind to a nick, and requires a single-stranded stretch longer than 25 nucleotides to do so (panel N). Finally, the single-stranded portion necessary for performing the unwinding of the 17 bp duplex can be as short as 39 nucleotides, since the linear substrate shown in panel P is unwound as effectively as the full length circular one.

A



B

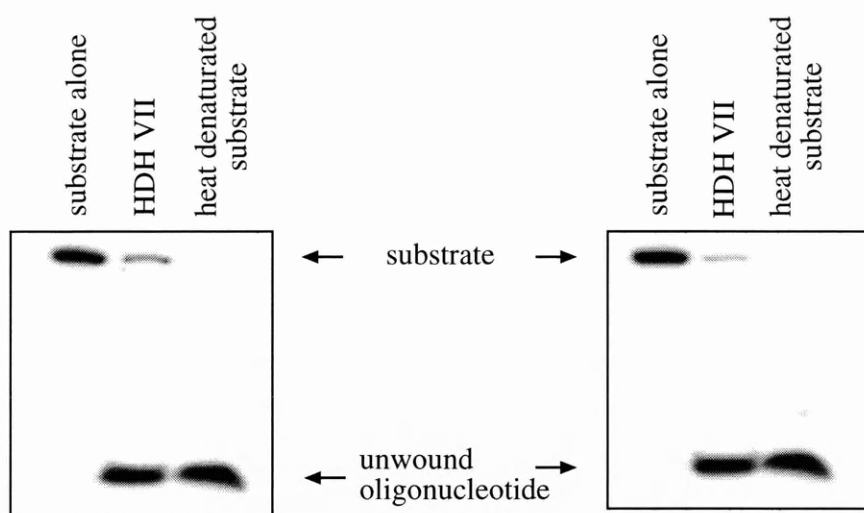
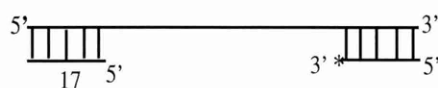


Figure 4.1.7. **Polarity of DNA Unwinding by HDH VII.** Panel A, substrate for 3'-5' polarity; Panel B, substrate for 5'-3' polarity. The top shows the structure of each substrate while bottom shows the autoradiogram of the relative gel. 0.15ng of pure HDH VII were used in this assay. Lanes 1 (in both panels) are substrates alone; lanes 2, the observed DNA unwinding of HDH VII; lanes 3, heat denaturated substrates.

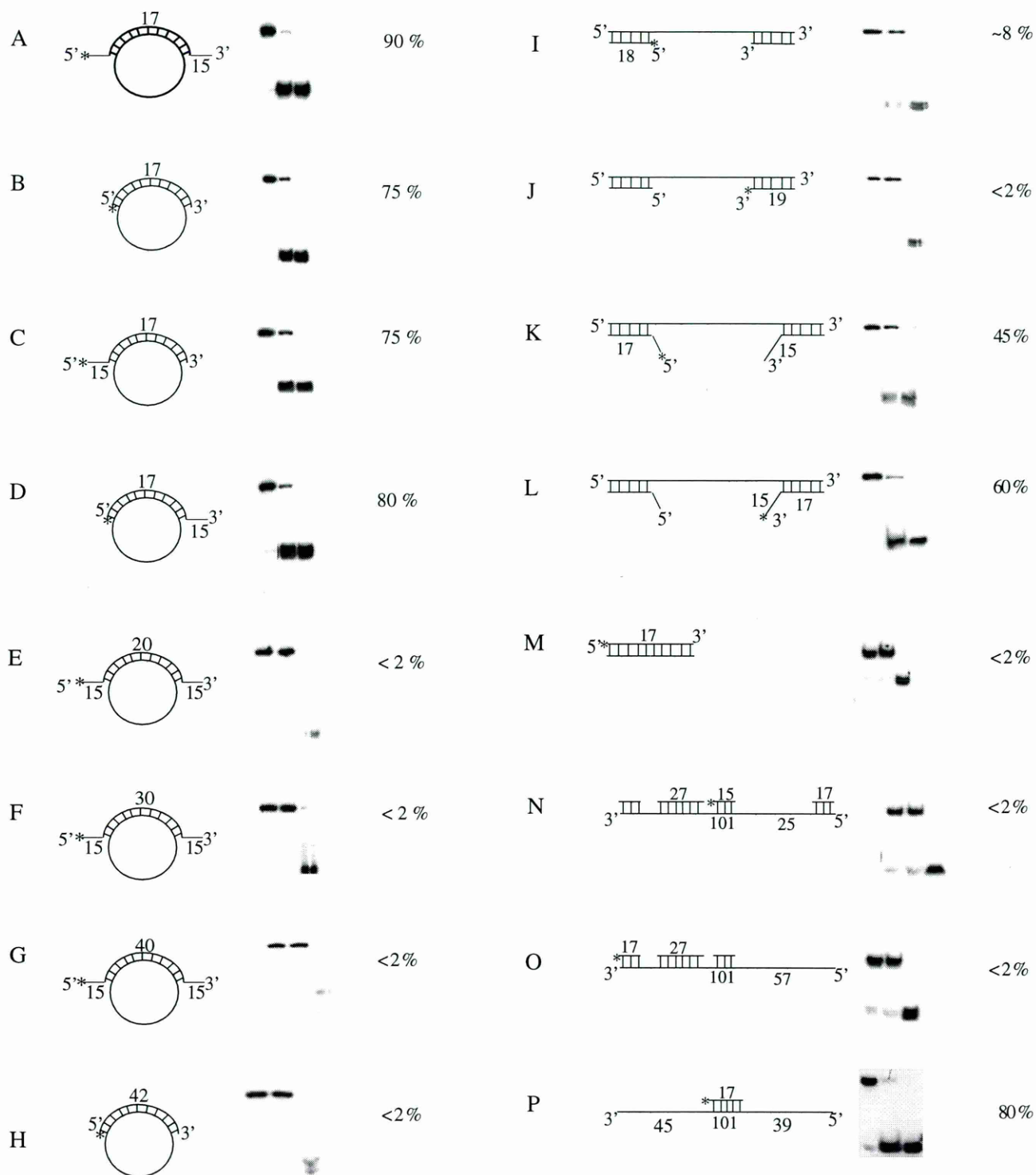


Figure 4.1.8. Reactivity of HDH VII With Various Substrates. The DNA unwinding observed for HDH VII in the presence of various substrates was quantitated. The structures of these substrates and the corresponding percentage of unwinding are shown in panels A through P.

4.1.9. Effect of hRPA on the Activity of HDH VII

The DNA unwinding activity of HDH VII is remarkably stimulated in the presence of the single-stranded DNA binding protein hRPA (see Figure 4.1.9 panel A). Quantitation of this effect, shown in panel B, and in graph form in panel C, shows an up-to 90-fold stimulation of the helicase activity of HDH VII. Such an effect is quite impressive since, *in vivo*, it could give rise to a 90-fold reduction in the time required by a given amount of HDH VII, or to a 90-fold reduction in the amount of enzyme required, to accomplish a given task.

4.1.10. The Stimulation of HDH VII by RPA is Species Specific

I compared the percentage of DNA unwinding observed for HDH VII in the presence of hRPA with that observed for the same enzyme in the presence of *E. coli* SSB. As shown in Figure 4.1.10, whereas a strong stimulation of DNA unwinding is observed in the presence of hRPA, no such effect is detected in the presence of *E. coli* SSB (compare lane 3 with lane 4, and lane 3 with lane 7). On the contrary, a slight inhibition of the activity of HDH VII is measured in the presence *E. coli* SSB due probably, to some sort of competition between the helicase and the SSB for the DNA substrate. These stimulatory/ inhibitory effects are presented more clearly in table 4.1.III.

4.1.11. Stimulation of the Activity of HDH VII by hRPA is non-Processive

The strong stimulation of the activity of HDH VII by hRPA led me to investigate its possible effects on the processivity of this enzyme. The results of these assays, shown in figure 4.1.11 A, indicate that the processivity of HDH VII is not affected at all by hRPA.

In another series of assays, I tested the effects of increasing concentrations of hRPA with a fixed amount of the helicase and, *vice versa*, those of increasing amounts of the helicase with a fixed amount of the SSB on the processivity of the enzyme. But in all cases the limited processivity of the enzyme was not affected (figure 4.1.11 panels B and C).

It is noteworthy that this strong stimulation operated by hRPA, on the activity of HDH VII, is totally absent on substrates with an annealed portion of more than 17 base pairs even in the presence of increasing concentrations of both helicase and SSB. That is to say that the stimulatory effect of hRPA is observed only on DNA substrates that sustain unwinding by HDH VII alone.

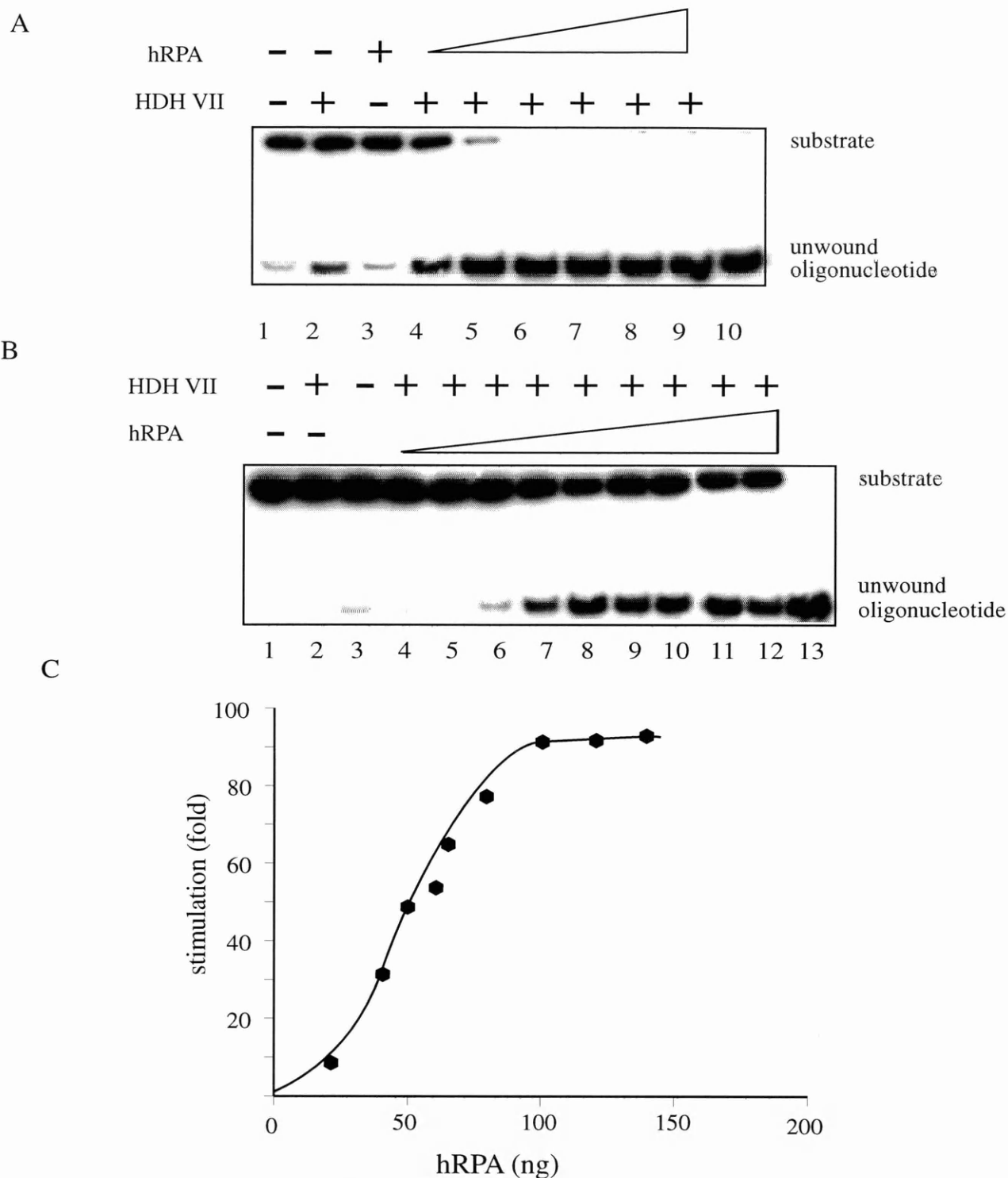


Figure 4.1.9. Effects of hRPA on the DNA Unwinding Activity of HDH VII.

Helicase assays were run in the presence of increasing amounts of hRPA. Panel A, qualitative assay: lane 1, substrate alone; lane 2, substrate with 0.03ng of enzyme ; lane 3, substrate with 30ng of hRPA ; lanes 4-9, substrate with 0.03ng of enzyme and 5ng, 10ng, 15ng, 20ng, 25ng, 30ng of hRPA respectively; lane 10, heat denatured substrate. Panel B, quantitative assay: lane 1, substrate alone; lane 2, substrate with approximately 1pg of enzyme; lane 3, substrate with 140ng of hRPA ; lanes 4-12, substrate with ~ 1pg of HDH VII and 20ng, 40ng, 50ng, 60ng, 70ng, 80ng, 100ng, 120ng, 140ng of hRPA respectively; lane 13, heat denatured substrate. Panel C, graph version of the data in panel B.

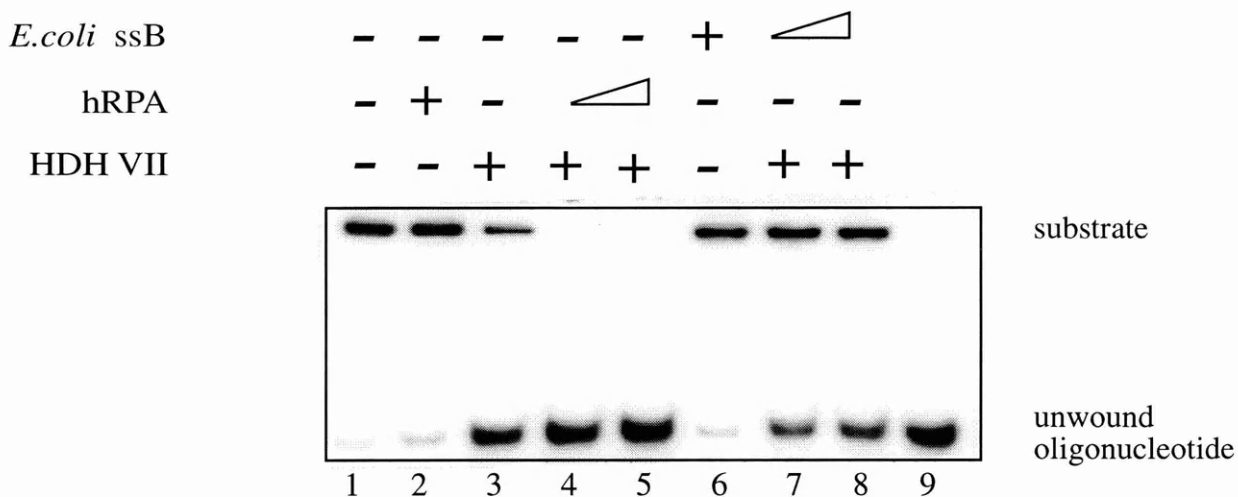


Figure 4.1.10. **Specificity of Stimulation by hRPA.** The helicase activity of HDH VII in the presence of hRPA was compared to the activity observed for it in the presence of *E.coli* SSB. Lane 1, substrate alone; lane 2, substrate with 80ng (0.64pmol) of hRPA ; lane 3, substrate with 0.06ng of HDH VII ; lanes 4 and 5, substrate with 0.06ng of HDH VII plus 40ng (0.32pmol),and 80ng (0.64pmol) of hRPA respectively; lane 6, substrate with 18ng (1.0pmol) of *E.coli* SSB ; lanes 7 and 8, substrate with 0.06ng of HDH VII plus 9ng (0.5pmol), 18ng (1.0pmol) of *E.coli* SSB respectively; lane 9, heat denatured substrate.

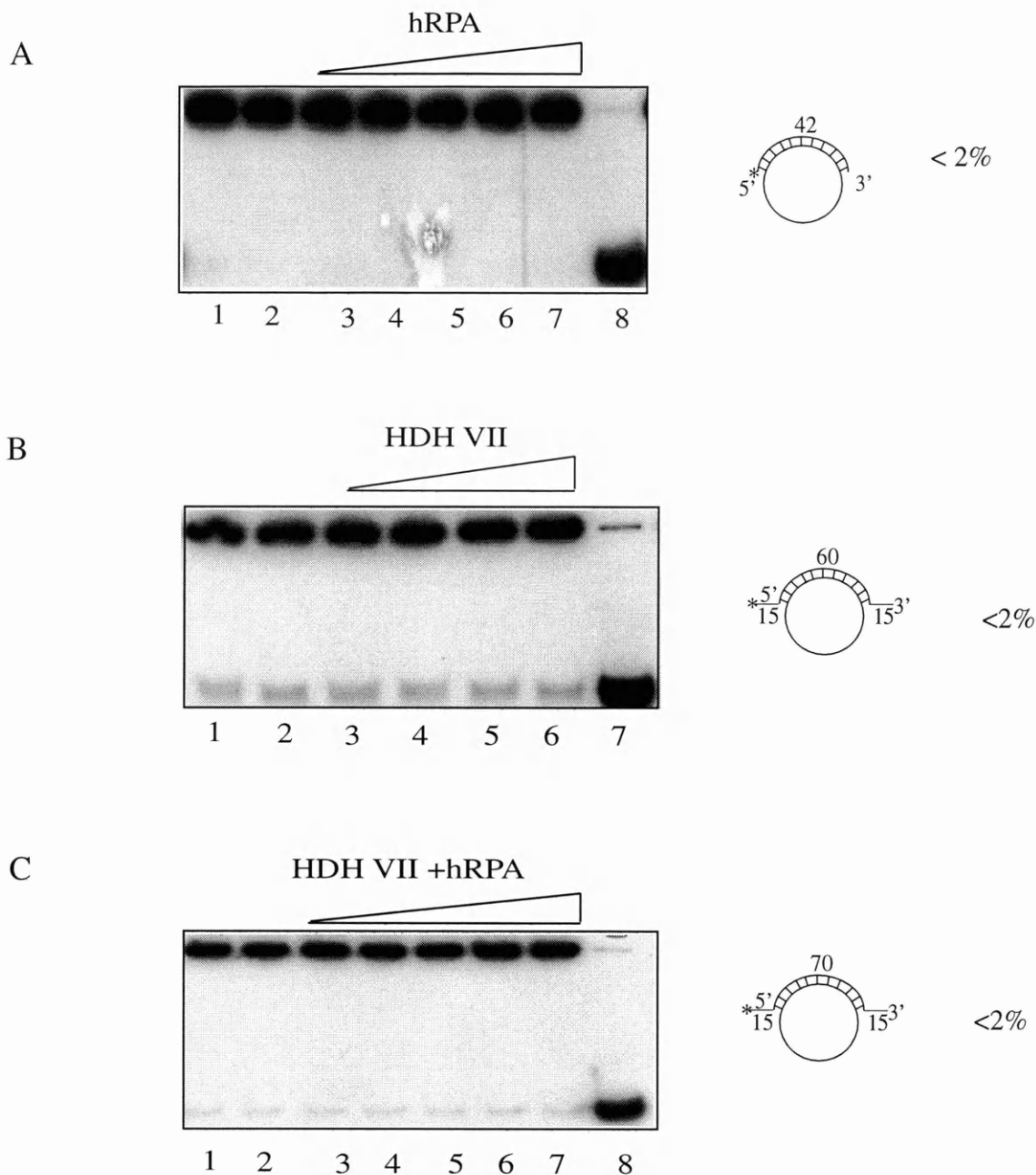


Figure 4.1.11: Effect of RPA on the processivity of HDH VII. Helicase assays were carried out in the presence of increasing amounts of hRPA, of HDH VII or of both. Panel A, increasing amounts of hRPA: lane 1, substrate alone; lane 2, substrate with 0.1 ng of HDH VII; lane 3, substrate with 0.1 ng of HDH VII and 50 ng hRPA; lane 4, substrate with 0.1 ng of HDH VII and 100 ng hRPA; lane 5, substrate with 0.1 ng of HDH VII and 150 ng hRPA; lane 6, substrate with 0.1 ng of HDH VII and 200 ng hRPA; lane 7, substrate with 0.1 ng of HDH VII and 250 ng hRPA; lane 8, heat denatured substrate. Panel B, increasing amounts of HDH VII: lane 1, substrate alone; lane 2, substrate with 0.1 ng of HDH VII; lane 3, substrate with 250 ng hRPA and 0.1 ng of HDH VII; lane 4, substrate with 250 ng hRPA and 0.5 ng of HDH VII; lane 5, substrate with 250 ng hRPA and 1.5 ng of HDH VII; lane 6, substrate with 250 ng hRPA and 3.0 ng of HDH VII; lane 7, heat denatured substrate. Panel C, increasing amounts of RPA and helicase: lane 1, substrate alone; lane 2, substrate with 0.1 ng of HDH VII; lane 3, substrate with 0.1 ng of HDH VII and 50 ng of hRPA; lane 4, substrate with 0.5 ng of HDH VII and 100 ng of hRPA; lane 5, substrate with 1.5 ng of HDH VII and 150 ng of hRPA; lane 6, substrate with 3.0 ng of HDH VII and 200 ng of hRPA; lane 7, substrate with 6.0 ng of HDH VII and 250 ng of hRPA; lane 8, heat denatured substrate.

Table 4.1.III. Comparison Between the Effects of hRPA and *E.coli* SSB on the Activity of HDH VII

Enzyme Species	Amount (ng)	% of DNA unwinding
HDH VII alone	0.06	40
hRPA alone	80 (0.64 pmol)	< 2
HDH VII + hRPA	0.06 + 40	~ 100
HDH VII + hRPA	0.06 + 80	~ 100
<i>E.coli</i> SSB alone	18 (1.0 pmol)	< 2
HDH VII + <i>E.coli</i> SSB	0.06 + 9	25
HDH VII + <i>E.coli</i> SSB	0.06 + 18	17

Table. 4.1.IV. Comparison of the Specific Activities of Some DNA Helicases of HeLa Cells

Enzyme	Spec. Activity (U/mg)	Stimulation by hRPA	Comments	References
HDH I	136,362	absent	-	165
HDH II	66,769	absent	Identical to the human Ku auto antigen	116
HDH III	40,400	N.D.**	-	167
HDH IV	51,074	slight	Identical to human Nucleolin	168
HDH V	20,416,000	N.D.**	-	169
HDH VI	1,200,000	absent	-	170
HDH VII	24,000,000*	strong	-	this work
HDH VIII	30,000	absent	Identical to G3BP	171
Rec. QL	1,090,000	inhibition	inhibition might be due to competition for substrate	unpublished
Mouse FM3A Helicase	5,000,000	absolute requirement	RPA enhances enzyme processivity	139

* Average of two preparations ** Not determined

4.1.12. HDH VII is a Hetero-trimer in Solution

I determined the native molecular weight of HDH VII by a combination of glycerol gradient sedimentation and gel filtration chromatography in buffer C according to Siegel and Monty and as described in materials and methods (see figure 4.1.12). From the results of the gel filtration (panels A and B) I calculated a Stokes radius of 36 Å, and from those of the glycerol gradient, (panels C and D), a sedimentation coefficient of 5.4 S was deduced in a plot of the S values of selected molecular weight markers versus their sedimentation positions on the gradient. Applying both parameters to the Siegel and Monty equation I obtained a native Mr of 95-kDa for HDH VII.

Such a molecular mass suggested a probable tri-meric conformation for HDH VII considering the presence of two protein bands in the region of 31-kDa observed in an SDS-PAGE analysis of the pure enzyme; bands, which as mentioned above, show different intensities when revealed by Coomassie Brilliant Blue staining (see gel in figure 4.1.1 panel B). A hypothetic tri-meric protein, composed probably of two equal subunits and a slightly larger third subunit, would fit with this data. Infact, estimation of the molecular weights of these protein bands by means of their Rf values gave a global molecular weight consistent with the proposal of a trimeric arrangement of the enzyme. Furthermore, when the fractions from the gel filtration chromatography were analyzed by SDS-PAGE, I observed the presence of the two closely migrating bands in the helicase-active fractions (figure 4.1.1. panel B). I therefore concluded that the DNA unwinding activity observed in the 95 kDa range in these two analyses is effectively due to the protein bands observed around 31 kDa and that these must associate in a tri-meric arrangement to constitute the active enzyme.

Determination of molecular mass, by gel filtration, of ~0.5 µg of HDH VII in the presence of 250 ng of hRPA showed no difference with the mass measured in the absence of this SSB, suggesting that the two proteins do not spontaneously form a stable complex *in vitro*. Possible interactions between HDH VII and hRPA, which resulted in the observed stimulation of the activity of the helicase, must therefore either be of a transient nature or the SSB may stimulate the unwinding activity, without participating in it, by acting exclusively at the DNA level.

HDH VII is a nuclear protein of relatively low abundance. Infact only 110 µg of enzyme were recovered from 150 g of cultured HeLa cells, corresponding to a mere 0.007% of the 1600 mg of total proteins extracted, before the ammonium sulphate precipitation. From the Mr in native form of the enzyme and the final yield obtained in the purification, I can estimate an abundance of approximately 30 000 to 50 000 molecules of HDH VII per cell.

Taken together, the results of the dependence of DNA unwinding on reaction time, the enzyme concentration, dependence and the low processivity of HDH VII clearly indicate

an enzyme that functions at extremely low enzyme/DNA substrate ratios, an enzyme that is probably endowed with an elevated rate of DNA unwinding and that is committed to a very limited task in the cell.

4.1.13. Identification of HDH VII By N-Terminal Microsequencing

Efforts aimed at the N-terminal microsequencing, in order to determine the amino acid sequence and also to confirm the three-subunit molecular arrangement of HDH VII, have so far been unsuccessful because the N-terminus appeared inaccessible to the Edman reagent. Therefore, a tryptic digestion of the enzyme was carried out at the laboratories of PRIMM Italia, and a data base search which compared the masses of the resulting peptides with those of similarly generated peptides revealed no match with fragments of any known human protein. This shows that HDH VII is, indeed, a novel human protein and further strengthens my opinion on the importance of this enzyme. Unfortunately, due to low peptide recovery of these tryptic fragments, it has not been possible to obtain any amino acid sequence data for this enzyme. Efforts are currently in progress to isolate a larger amount of this enzyme from HeLa cells to guarantee successful determination of the amino acid sequence. Figure 4.1.13 shows a mass spectrogram of elution of the tryptic digest fragments of HDH VII.

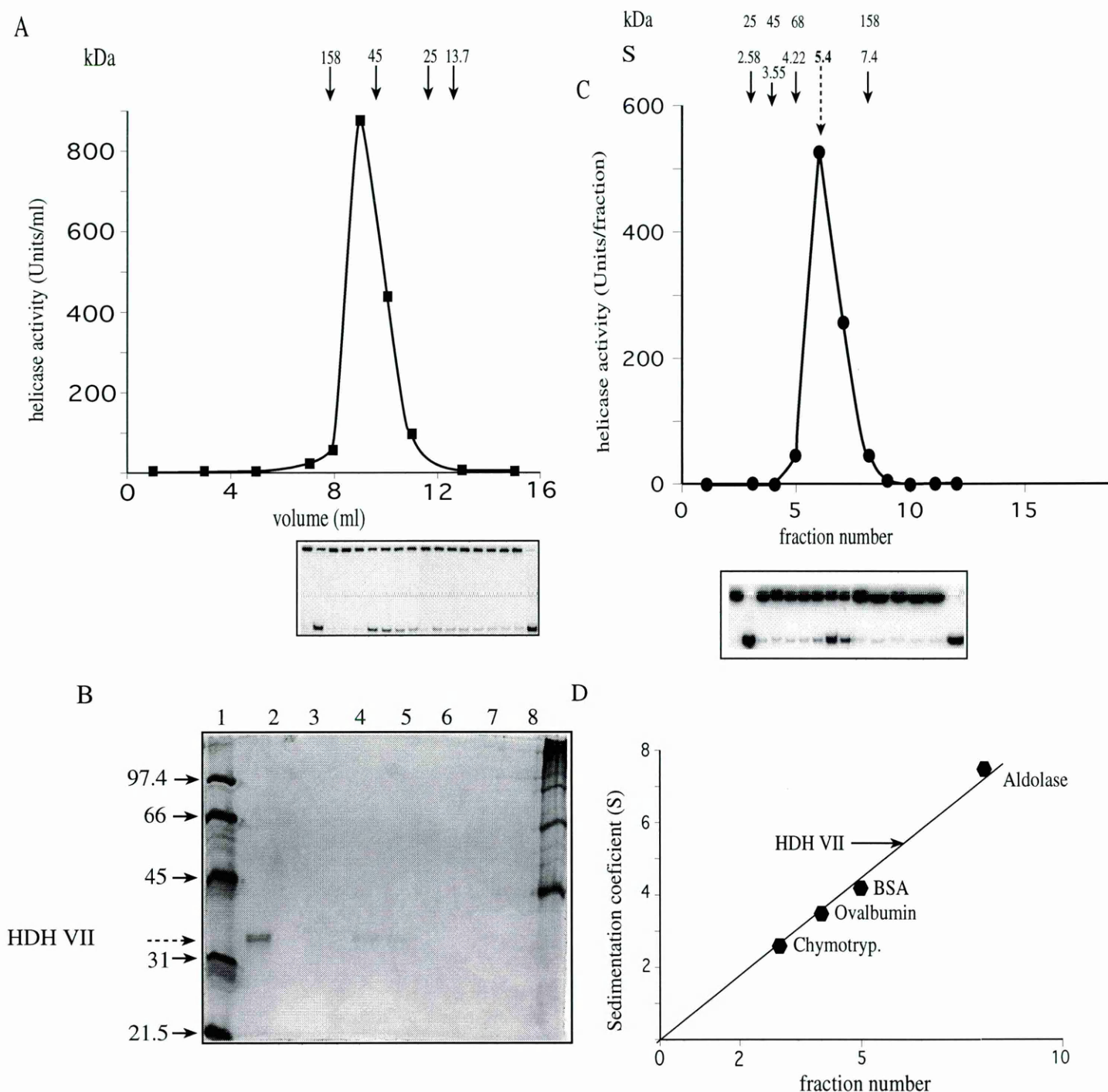


Figure 4.1.12. Determination of the Molecular Weight of HDH VII by Gel Filtration Chromatography and Glycerol Gradient Sedimentation. Panel A, quantitation of the helicase activity in the gel filtration fractions. The elution volumes of selected markers: Aldolase (158kDa), Ovalbumin (45kDa), Chymotrypsinogen (25kDa) and Ribonuclease (13.7kDa) are indicated. Panel B, relative SDS-PAGE analysis. Lane 1, low-range markers; lane 2, sample loaded on the column; lanes 3-8, fractions 7-12 respectively. The migration of HDH VII is indicated. Panel C, quantitation of helicase activity in the glycerol gradient fractions. The masses and S values of selected markers are shown. The sedimentation position of HDH VII is indicated at the extrapolated S value. The relative autoradiograms are shown. Panel D, plot of S values versus the sedimentation positions of the markers. The position of HDH VII is indicated with the arrow.

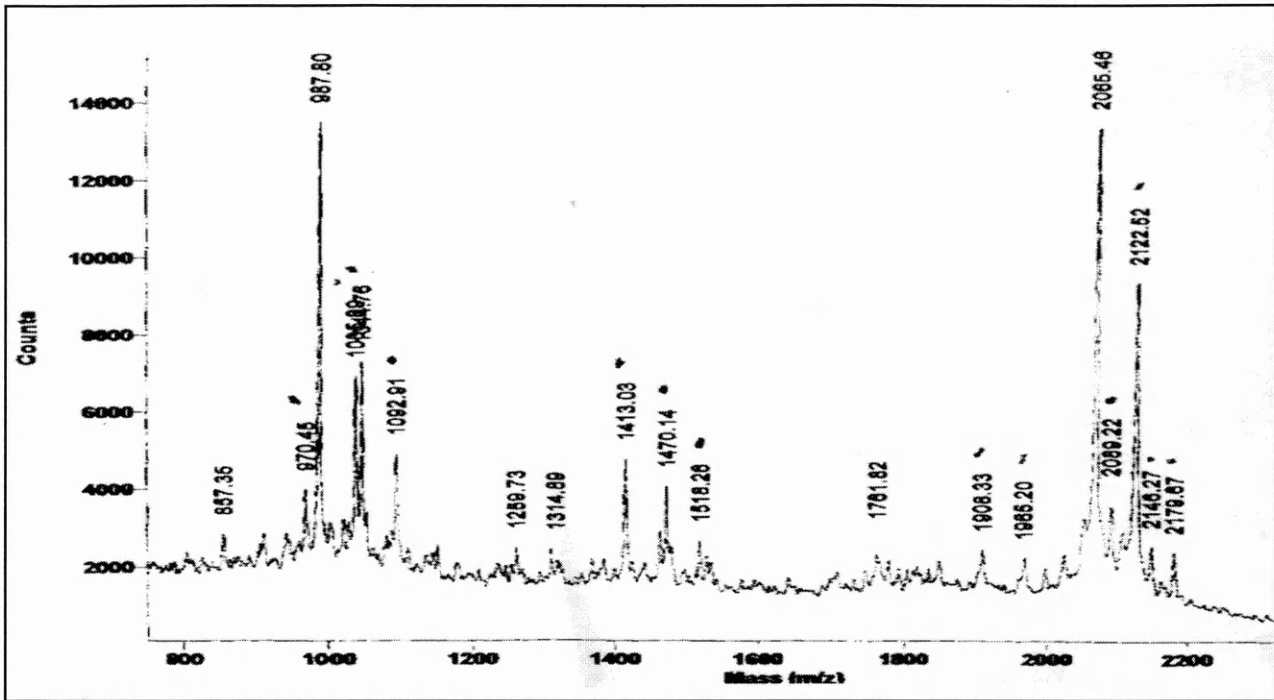


Figure 4.1.13. **Mass Spectrogram of the tryptic digestion of HDH VII:** Computer search and subsequent comparison of the mass of these peptides revealed no match with similarly generated fragments of any known human protein, indicating that HDH VII is a novel human protein.

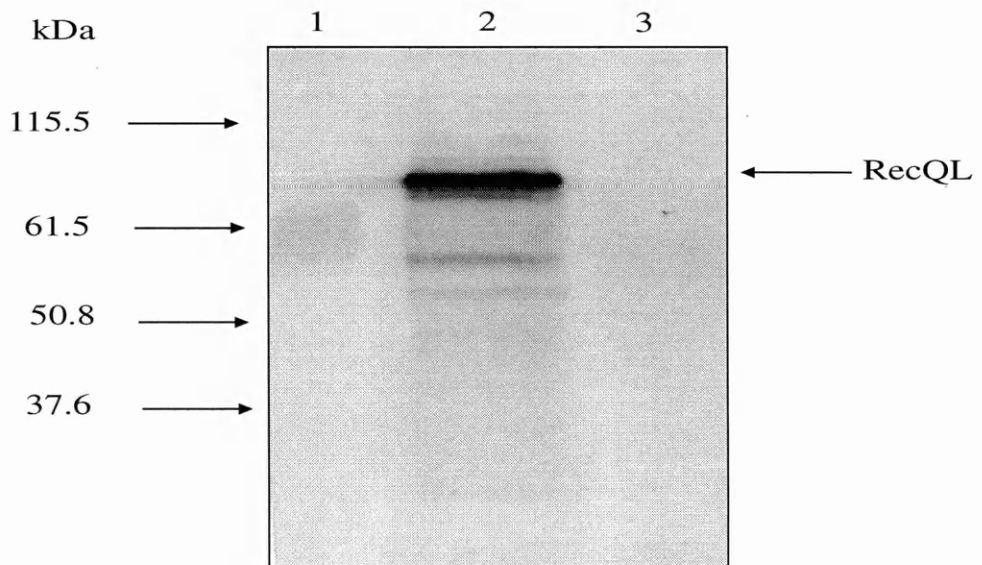


Figure 4.1.14 **Western blotting with anti-RecQL antibody:** western blot analysis confirms that HDH VII is not immunologically related to RecQL since it is not recognized by a polyclonal antibody against this protein and it is not contaminated by RecQL (lane 3); lane 2, RecQL; lane 1Mr markers.

4.2. Functional Properties of the Recombinant Ku/HDH II subunits

The Ku protein is a heterodimer of 83-kDa and 70-kDa subunits that possesses dsDNA binding and unwinding properties. Despite the fact that the genes encoding the separate subunits of this protein are located on different chromosomes, these component subunits could not be separated by any physical methods except by the action of denaturing agents. The availability of the recombinant Ku subunits allowed me to investigate the possibility of ascribing these *in vitro* activities to either subunit.

4.2.1. Purification and Refolding of the Separate Subunits of Recombinant HDH II/Ku

The recombinant subunits of the HDH II/Ku protein accumulated in inclusion bodies were isolated by standard methods described in materials and methods. These were further subjected separately to gel filtration on Sephacryl S300 resin and the very clean fractions were pooled and used for the renaturation procedure. Earlier attempts to renature these subunits separately by dialysis were unsuccessful since they showed intrinsic tendency to precipitate. However, when these separate subunits of HDH II/Ku (50 ml of Sephacryl S300 purified fractions of each subunit in Buffer G), as well as an equimolar mixture of these, were treated individually by addition of the non-detergent solubilising and stabilising agent NDSB 195 (dimethyl ethylammonium propane sulfonate) (186-188) at a final concentration of 0.2M, and dialyzed at 4°C versus 8 changes of 2000 ml of buffer R, each of the separate subunits remained in solution during dialysis and renaturation could thus be achieved. (An earlier attempt at refolding using NDSB 195 at a final concentration of 0.1M had resulted in slight precipitation of the separate subunits during dialysis). NDSB is a non-detergent zwitterionic molecule and hence dialyses easily; it is however, a significantly larger molecule than guanidine and one might then expect that the latter will dialyse out before NDSB. The dialyzed samples were centrifuged at 10 000 rpm at 4°C in a Sorvall super speed 34 rotor for 20 minutes and the supernatants were used either directly or after one further purification step, for DNA binding and helicase assays.

The refolded separate subunits were individually subjected to a further purification step by MonoQ anion exchange chromatography (FPLC). After extensive washing of the column, bound material was eluted in each case with 20 column volumes of 0.1M - 1M NaCl linear gradient. In each case the subunit eluted at approximately 0.25 M salt.

The reconstituted heterodimer was further purified by dsDNA-Sepharose affinity chromatography. Figure 4.2.1, panel A, B and C show the various analysis of these Ku subunits.

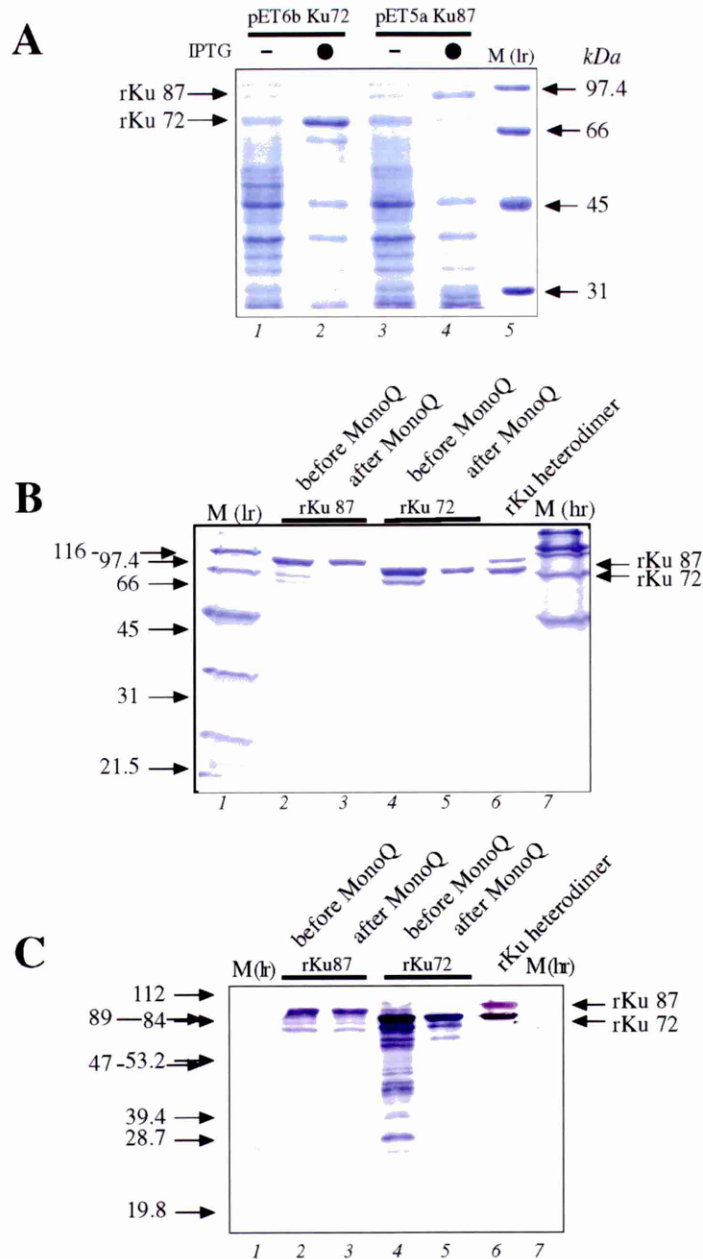


Figure 4.2.1: Purification of the separate recombinant HDH II/Ku subunits. Panel A: Expression of Ku subunits in BL 21 (DE3) (plysS) *E.coli*. Total cell extract corresponding to 50 μ l uninduced or IPTG-induced cultures were analysed on SDS-10% polyacrylamide gel. Lane 1, pET66Ku72 uninduced; lane 2, pET66Ku72 induced; lane 3, pET5aKu87 uninduced; lane 4, pET5aKu87 induced; lane 5, molecular weight markers with corresponding mass indicated on the right hand margin. Proteins are visualised by coomassie brilliant blue staining.

Panels B and C: SDS-PAGE and Western blot analyses of refolded recombinant Ku and subunits. Approximately 5 μ g of each protein sample were analysed on SDS-10% polyacrylamide gel and visualised by coomassie brilliant blue staining (panel B) or revealed by Western blotting (panel C). Lanes 1, Low range molecular weight markers; lanes 2 and 3, rec.87 kDa subunit before and after MonoQ chromatography respectively; lanes 4 and 5, rec.72 kDa subunit before and after MonoQ; lanes 6, recombinant heterodimer; lanes 7, high range markers.

4.2.2. The Helicase Activity of HDH II/Ku Resides in the 70 kDa Subunit

The renatured separate subunits of HDH II/Ku were assayed for the *in vitro* activities (band-shift and helicase) to determine whether these could be associated with either subunit. As shown in figure 4.2.2 panel A, the 70 kDa subunit possesses a helicase activity comparable to that of the heterodimer, whereas the 83 kDa subunit shows no detectable helicase activity. Neither subunit shows any appreciable capacity to retard the mobility of duplex DNA, indicating that neither of them alone has the ability of the native HDH II/Ku to bind to the duplex ends (figure 4.2.2 panel B). Conversely, the 70 kDa subunit alone maintains a measurable affinity for ssDNA, comparable to that of native and recombinant HDH II/Ku (figure 4.2.2 panel C) in agreement with the maintenance of the helicase activity in this subunit, since most known helicases bind initially to the ss portion of the substrate. These findings suggest that, while the helicase activity ascribed to HDH II/Ku resides only in the 70 kDa subunit, the DNA end-binding capacity requires the presence of both subunits in the heterodimeric form. A comparative quantitation of the activities of the different forms of HDH II/Ku is reported in Table 4.2.I. Before analyzing in detail the catalytic and binding constants for functional properties of the different molecular forms, I investigated their subunit composition.

4.2.3. Quaternary Structure of the Renatured Molecules

I determined the native Mr of the refolded separate subunits of HDH II/Ku as well as that of a post-refolding equimolar mixture of these, by a combination of glycerol gradient sedimentation (15% - 35%) and gel filtration chromatography in buffer B as described in materials and methods to investigate whether the Ku heterodimer could be reconstituted by mixing equimolar amounts of the separately refolded subunits. As shown in figures 4.2.3 A and B, the helicase activity of the renatured 70 kDa subunit alone showed a native Mr correspondent to the one observed in SDS-PAGE; in the post-refolding equimolar mixture the same Mr was measured for helicase activity, whereas in the reconstituted heterodimer the activity showed an Mr corresponding to that of the sum of the Mr of the two subunits (figure 4.2.3 A). SDS-PAGE analysis of the gel filtration experiments showed that the 83 kDa subunit, whether renatured alone or in the post-refolding mixture, eluted at a volume close to that of the reconstituted heterodimer (figure 4.2.3 B). I therefore concluded that the refolded 70 kDa subunit of HDH II/Ku remained as a monomer in solution, even in the presence of equimolar amounts of the other subunit, and that the heterodimer could not be reconstituted by mixing and incubating equimolar amounts of the renatured subunits. Furthermore, the 83 kDa subunit, when renatured alone, formed homodimers that did not spontaneously exchange with the renatured 70 kDa subunit added subsequently (see Fig. 4.2.3 B).

A more sensitive comparative quantitative analysis of the functional properties *in vitro* of the different molecular forms was then performed.

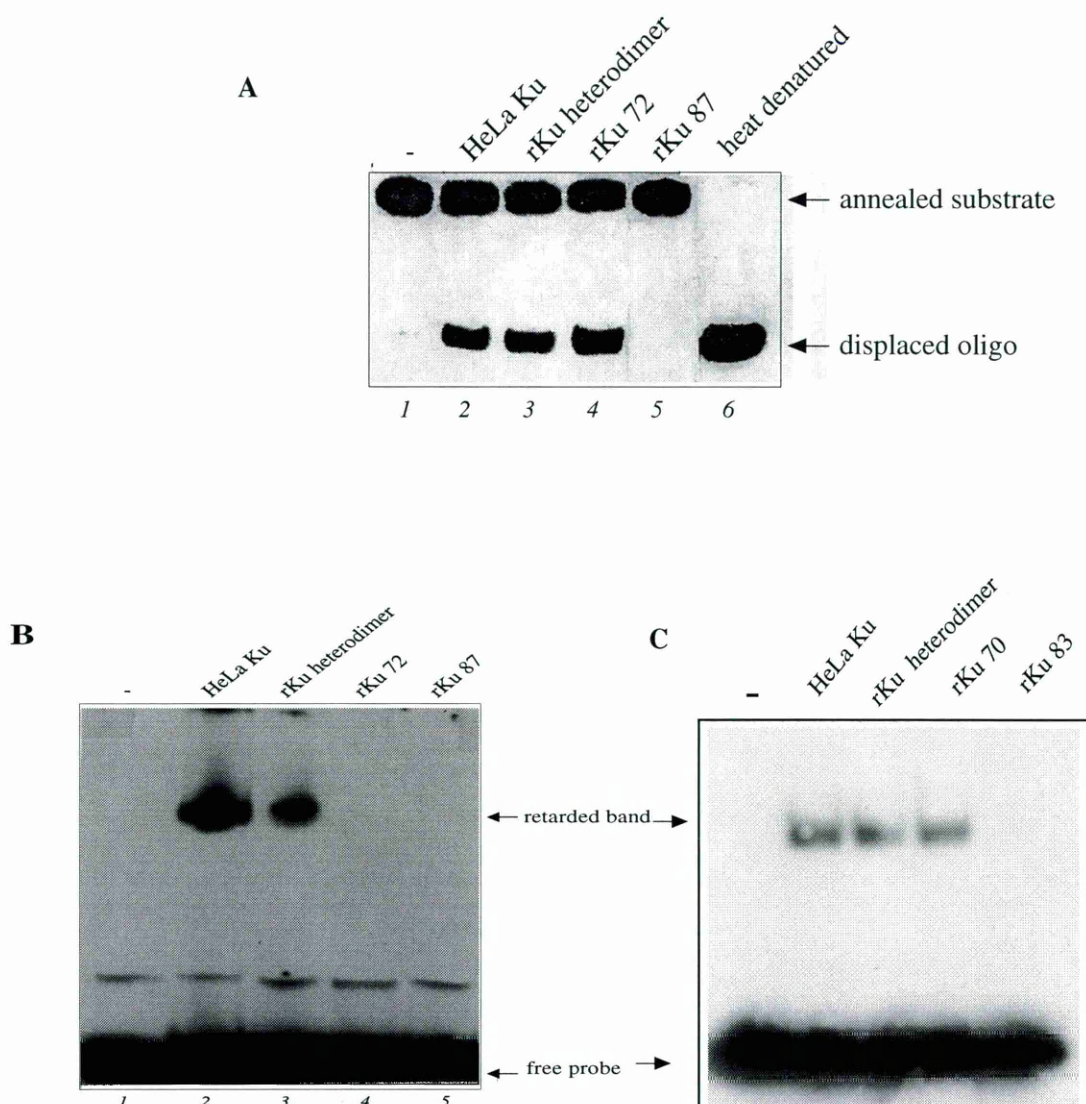


Figure 4.2. 2. Helicase and band shift assays for the Different Molecular Forms of HDH II/Ku.

Panel A: Helicase assay: lane 1, substrate alone, lanes 2-5: substrate with 5 ng HeLa purified HDHII/Ku, 11 ng recombinant heterodimer, 24 ng recombinant 70 Kda subunit, 80 ng recombinant 83 kDa subunit, respectively; lane 6: heat denatured substrate.

Panel B: Band shift assay: lane 1, DNA probe without protein; lanes 2-5, probe with 15 ng HeLa purified HDH II/Ku, 44 ng recombinant heterodimer, 96 ng recombinant 70 kDa subunit and 320 ng recombinant 83 kDa subunit, respectively.

Panel C: Band shift assay with ssDNA: lane 1, ssDNA probe without protein; lanes 2-5,

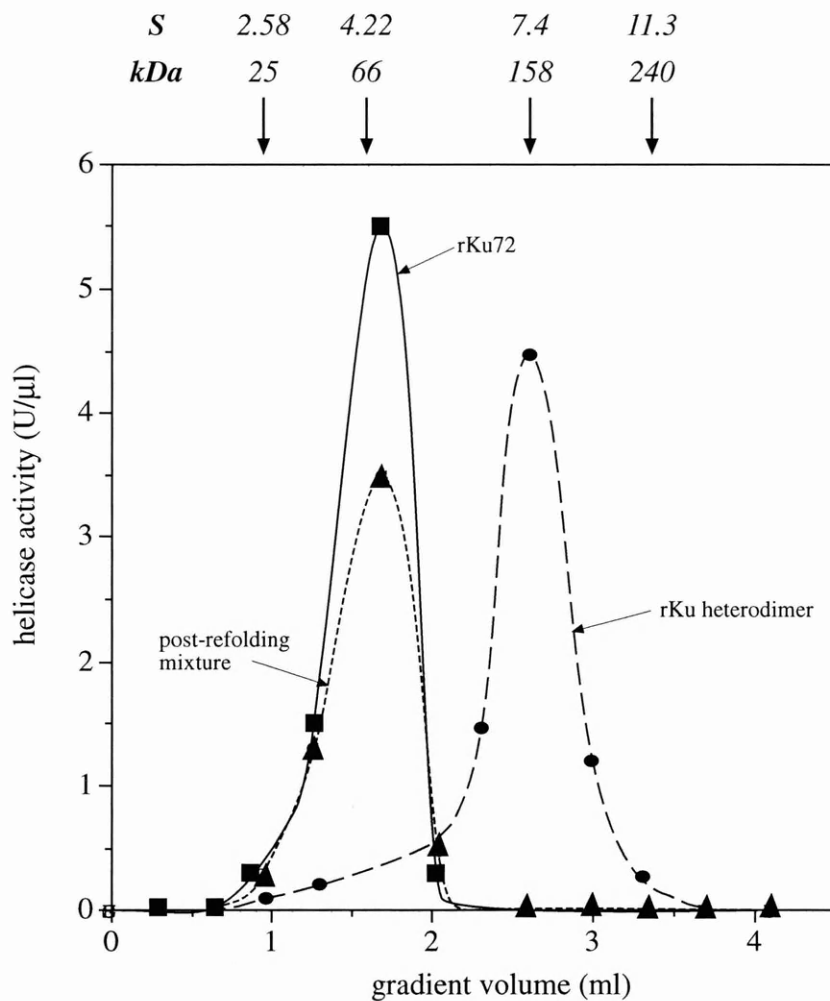


Figure 4.2. 3 A: Det. of Molecular Weight by Glycerol Gradient Sedimentation. This figure shows the distribution of molecular weights as monitored by helicase assay with fractions of the gradient on these molecular species. The position and S values of four selected molecular weight markers (chymotrypsinogen A, bovine serum albumine, aldolase and catalase) are indicated with arrows.

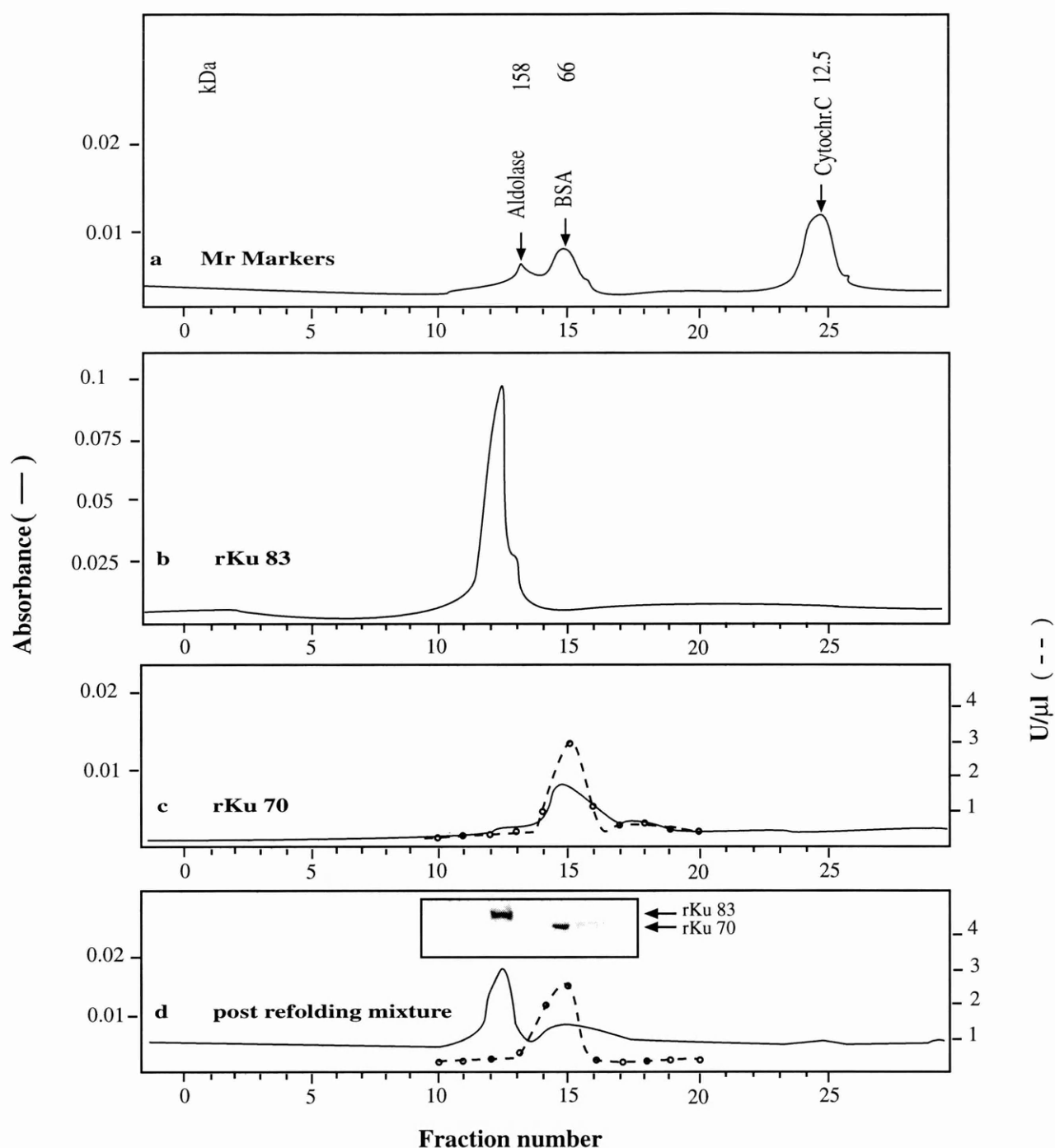


Figure 4.4.3 B. **Molecular Weight Determination by Gel Filtration Chromatography**

The refolded separate subunits of HDH II/Ku as well as a post-refolding equimolar mixture of these were separately subjected to gel filtration chromatography on a Superdex 75 (Pharmacia) 24 ml column (FPLC), as shown in the figure; 0.5 ml fractions were collected: a, selected Mr markers (Aldolase, BSA, Cytochrome C); b, rKu 83 alone; c, rKu 70 alone; d, post-refolding mixture of the separate subunits.

4.2.4. Substrate Concentration Dependence of Helicase Activity and Determination of K_m for the Various Forms of HDH II/Ku and Subunits

The various forms of HDH II (HeLa purified and the reconstituted recombinant) and the refolded separate subunits were assayed for helicase activity in the presence of increasing amounts of substrate, and DNA unwinding was evaluated as described in materials and methods. Figure 4.2.4.A shows a direct plot of DNA unwinding against substrate concentration relative to this assay. I observed a linear dependence of the helicase activity on the substrate concentration for all species assayed except the 87 kDa subunit which, as mentioned above, does not show any DNA unwinding activity. Figure 4.2.4.B shows the double reciprocal (or lineweaver Burk) plot to determine the K_m values relative to these enzyme species. It is interesting to observe that the curves relative to the helicase-active species all intersect the x-axis at the same point but intersect the y-axis at different points indicating differences only in their relative rates of DNA unwinding. I therefore concluded that these three enzyme species have the same affinity for the helicase substrate (K_m of 0.5nM) but different V_{max} values when expressed as mmol of substrate unwound $\times \text{mol}^{-1}$ of enzyme $\times \text{min}^{-1}$ (namely, 2.1 for the natural Ku form, 1.7 for the recombinant form, and 0.4 for the 70-kDa subunit).

4.2.5. Protein Concentration Dependence of DNA Binding and Determination of K_d for the Various Forms of HDH II/ Ku

The DNA binding ability, as assayed by electrophoretic mobility gel retardation, is essentially observed only for the native heterodimeric form of HDH II/Ku and, albeit at a markedly reduced level, for the recombinant heterodimer. The 70-kDa subunit exhibits only traces of DNA binding at very high protein/DNA ratios, whereas the 83-kDa subunit shows no detectable DNA binding in the range of assayed protein concentrations. Conversely, the ability to bind ssDNA, that in the native Ku form is approximately one-hundredth that for duplex DNA, is reconstituted almost completely in the recombinant heterodimer and (not surprisingly, as seen above) in the 70-kDa subunit, i.e. the moiety where the unwinding capacity resides. I then determined the protein concentration dependence for the DNA binding activity of the two heterodimeric forms (HeLa purified and recombinant) of HDH II/Ku. Figure 4.2.5 shows a linear dependence of DNA binding on protein concentration up to 5 ng for the HeLa-purified enzyme, with an apparent K_d for this species of 1.0 nM. The recombinant HDH II/Ku heterodimer showed, as pointed out earlier, only 1.8% of the DNA binding activity observed for the HeLa-purified enzyme (see table 4.2.I), and the estimated value of K_d for the reconstituted recombinant molecule is about 0.05mM.

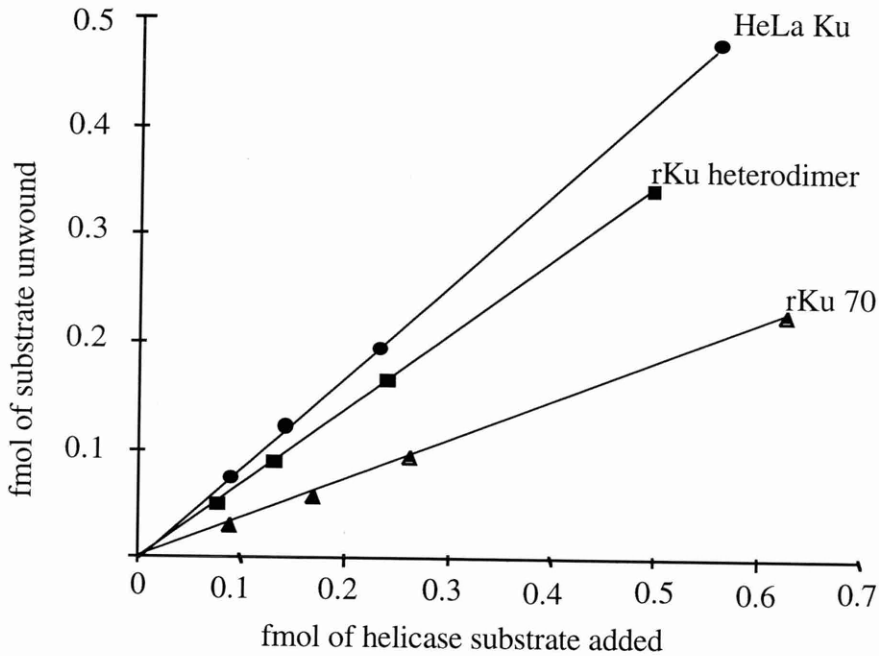
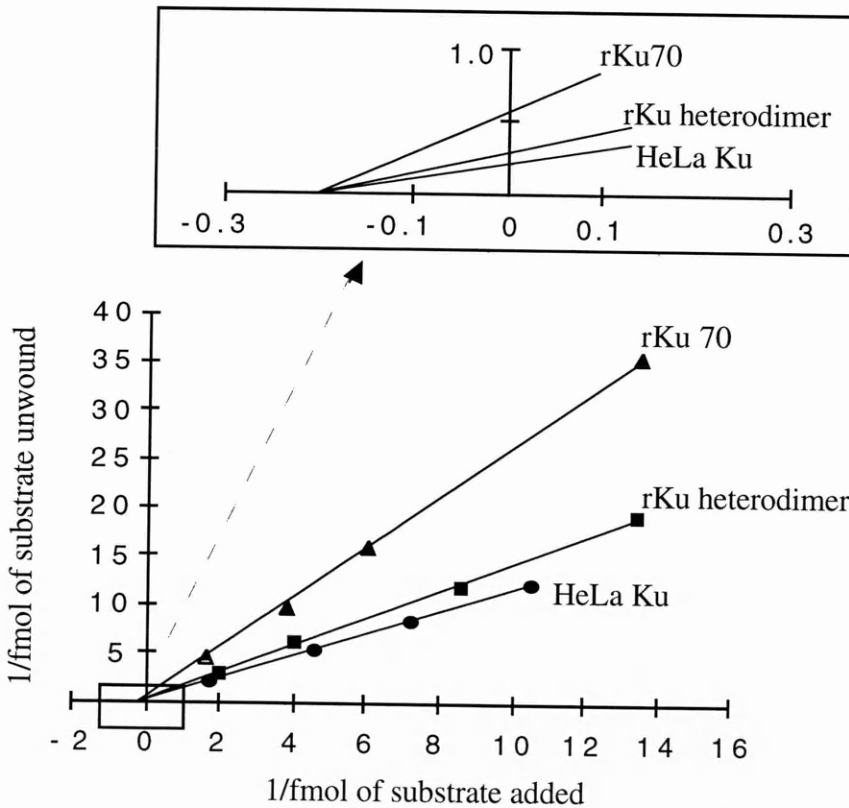
A**B**

Figure 4.2.4. Dependence of Helicase Activity on Substrate Concentration for the Various Forms of HDH II/Ku.

Helicase activity was quantitated in the presence of increasing amounts of helicase substrate using 11 ng of each of the various active species of enzyme.

Panel A: Direct plot of DNA unwinding versus substrate concentration.

Panel B: Double reciprocal plot from which K_m and V_{max} values are calculated. The box shows an enlargement of the intersect area

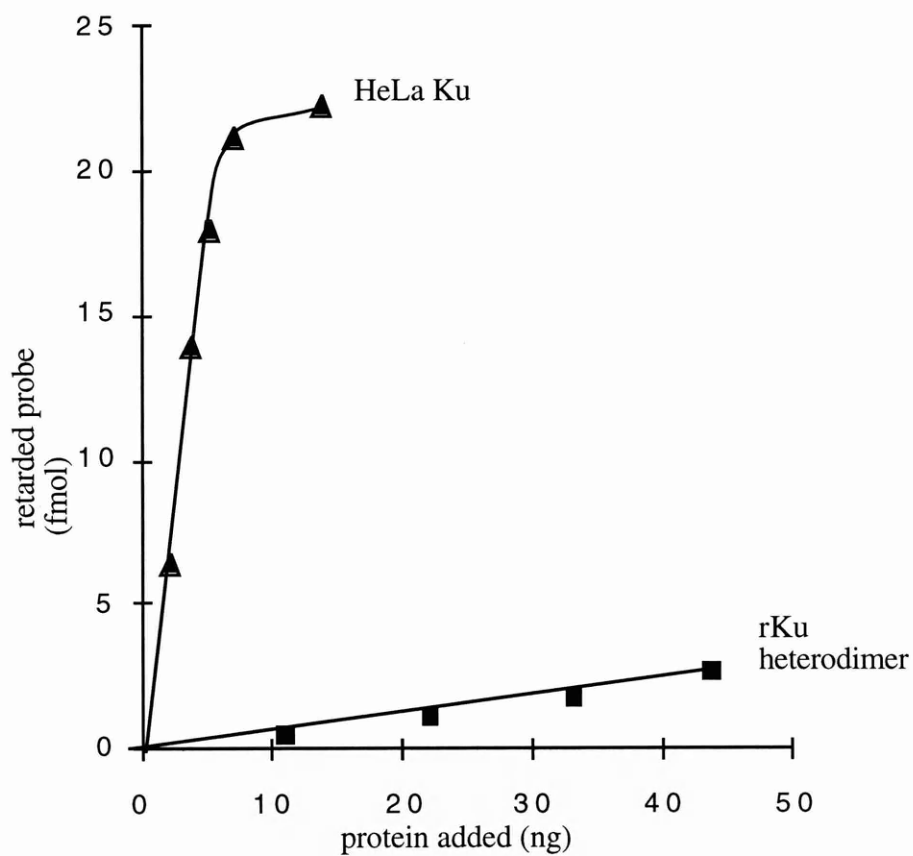


Figure 4.2.5. **Dependence of DNA Binding on Protein Concentration.**

Duplex DNA end-binding was measured for the two heterodimeric forms of HDH II/Ku (HeLa purified protein and recombinant heterodimer) in the presence of increasing amounts of either protein species (the insets report the corresponding autoradiographs).

4.2.6. Dependence of DNA Unwinding on ATP and Determination of the Relative K_M

DNA unwinding was measured for the helicase-active forms of HDH II/Ku in the presence of increasing amounts of ATP and shown in figure 4.2.6 panels A, B and C. Evaluation of the DNA unwinding at these ATP concentrations showed differences in the affinities of these enzyme species for ATP with estimated K_M values, for this co-factor, of 11.7 mM for HeLa-purified HDH II/Ku, 7.7 mM for the recombinant heterodimer and 4.4mM for the 70-kDa subunit (figure 4.2.7 A and B). As I have pointed out earlier, these various forms of HDH II/Ku showed the same affinity for the helicase substrate but their relative V_{max} of DNA unwinding varied (see figure 4.2.4 B). The differences in the affinities for ATP, observed in this experiment, are in perfect agreement with the differences observed earlier in their DNA helicase activities, and are direct consequences of the different abilities of these various forms of HDH II/Ku to bind and efficiently hydrolyze ATP.

4.2.7 ssDNA-Dependent ATPase Activity of the Different Molecular Forms

The various species of HDH II/Ku as well as the separate subunits were assayed for ssDNA-dependent ATPase activity, as described in materials and methods, in the presence of 100 μ M ATP. For all the species tested ATP hydrolysis was observed in the assay conditions, namely at the level of 1.12 μ mol ATP/mg for HeLa-purified Ku, 0.9 μ mol/mg for recombinant Ku heterodimer, 0.3 μ mol/mg for recombinant Ku 70 and 0.11 μ mol/mg for recombinant Ku 83 (see also Table 4.2.I). As shown above, ATP-dependent DNA unwinding ability had been observed for all these species except the 83 kDa subunit. The presence of this functionally unexplained ATPase activity in the large subunit agrees with the described presence of a putative ATP binding site in the same molecule. Table 4.2.II summarizes the catalytic and binding constants as a duplex and ssDNA binding protein, helicase and ATPase of the different forms of Ku.

Table 4.2.I: Quantitation of The *in vitro* Activities of Recombinant HDH II/Ku Heterodimer and Subunits, and Comparison With Those of HeLa Purified HDH II/Ku Heterodimer

Species assayed	DNA-binding *U/mg	% relative to HeLa Ku	Helicase activity **U/mg	% relative to HeLa Ku
HeLa HDH II /Ku	3600000	100	170000	100
Rec. HDH II /Ku	65000	1.8	150000	88.2
Rec. HDH II /Ku 87	<10	<0.01	<10	<0.05
Rec. HDH II /Ku 72	~780	~0.02	76000	44.7

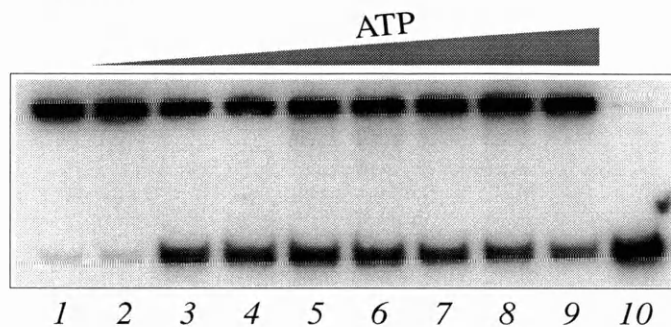
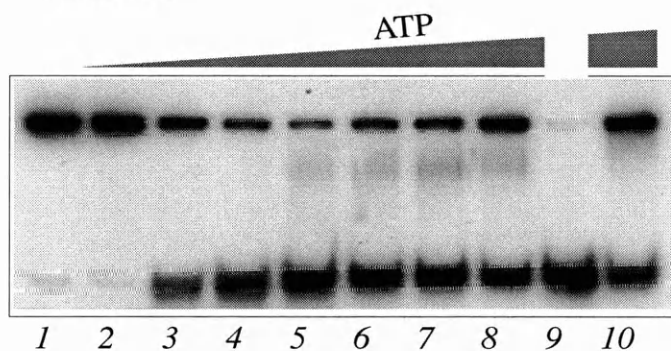
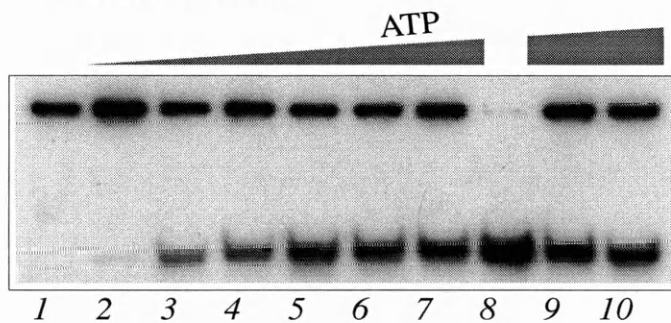
* 1 unit of DNA binding activity is the amount of enzyme that retards 1 fmol of DNA probe in a gel shift assay

** 1 unit of helicase activity is the amount of enzyme that unwinds 1% of substrate in 1 min (30% in 30 min) at 37°C in the linear range of enzyme concentration dependence

Table 4.2.II. Catalytic and Binding Constants of HDH II/Ku

Enzyme species	K _d for duplex 25-mer	K _m for helicase substrate	V _{max} as helicase (mmol of substrate unwound xmol of enzyme ⁻¹ x min ⁻¹)	K _m for ATP	V _{max} for ATP (mol of ATP hydrolyzed x mol enzyme ⁻¹ x min ⁻¹)
HeLa HDH II/Ku	2.0 nM	0.5 nM	2.1	11.7 mM	5.9
Rec HDH II/Ku	0.1mM	0.5 nM	1.7	7.7mM	4.8
Rec HDH II/Ku 87	>20 mM	>10.0 mM	< 0.0001	N.D.	0.7
Rec HDH II/Ku 72	~10 mM	0.5 nM	0.4	4.4 mM	0.3

N.D: not determinable

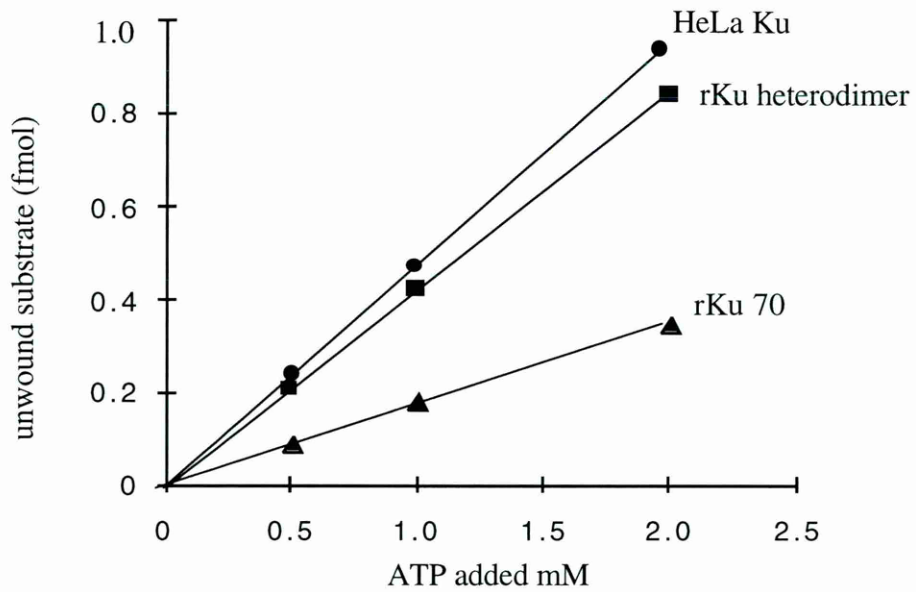
A**rKu 72****B****HeLa Ku****C****rKu heterodimer**

Panel A: Recombinant 70 kDa subunit; lane 1: no enzyme; lanes 2 to 9: assays with 0, 0.5, 1, 2, 3, 4, 5 and 6 mM ATP respectively; lane 10: heat denatured substrate.

Panel B: HeLa Ku; lane 1: no enzyme; lanes 2 to 8: assays with 0, 0.5, 1, 2, 3, 4 and 5 mM ATP respectively; lane 9: heat denatured substrate; lane 10: assay with 6 mM ATP.

Panel C: Recombinant Ku heterodimer; lane 1: no enzyme; lanes 2 to 7: assays with 0, 0.5, 1, 2, 3 and 4 mM ATP respectively; lane 8: heat denatured substrate; lanes 9 and 10: assays with 5 and 6 mM ATP respectively

A



B

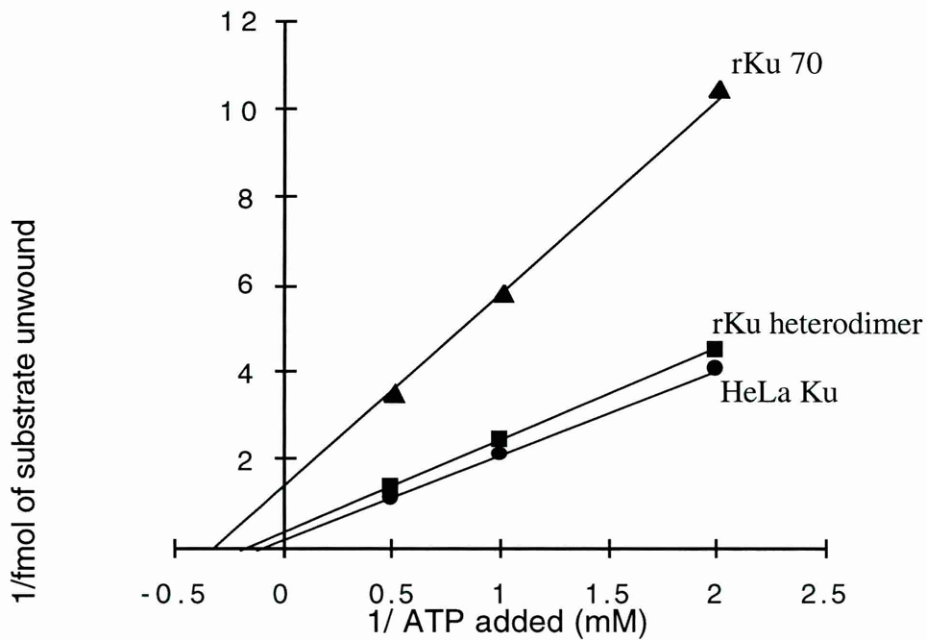


Figure 4.2.7. Determination of the K_m for ATP for the Helicase Activity of the Various Forms of HDH II/ Ku.

Panel A: Direct plot of dependence of helicase activity on ATP concentration determined from the data of the results shown in figure 6. The K_m values which these enzymes species exhibit for ATP are calculated from the double reciprocal plot in panel B.

4.2.8. Effect of Phosphorylation on the Helicase Activity of Recombinant HDH II/Ku

The DNA-dependent protein kinase of HeLa cells is a serine/threonine protein kinase that phosphorylates factors involved in several cellular processes (189). The Ku protein has often been reported to co-purify with the 350 kDa catalytic subunit of this DNA-dependent protein kinase, and has been reported to be involved in the phosphorylation activity of the kinase. Ku has, therefore, been identified as the DNA binding component of this protein kinase complex (190). However, during this phosphorylation both the Ku protein and the catalytic subunit get phosphorylated and, therefore, Ku serves, not only as the DNA binding component in the complex, but also as substrate for the kinase activity. Since the Ku protein expressed in *E.coli* lacked post-translational modifications such as phosphorylation, and since I successfully separated the two components of this kinase complex by anion exchange chromatography, on a Mono Q (FPLC) column, I explored the possible effects of this modification on the *in vitro* activities of HDH I/Ku.

4.2.9. Purification of the Catalytic Subunit of DNA-PK

Purification of this enzyme went *pari passu* with that of HDH VII with which it co-purified through the first two steps up to the strong anion exchange chromatography on Mono Q (FPLC), on which HDH VII did not bind while DNA-PK eluted at 0.26 M of a linear 0.1 M to 1.0 M NaCl gradient. From an initial 1.6 g of total proteins extracted, 4.5 mg of proteins containing kinase activity was pooled at this step. This pool was further purified on a dsDNA-cellulose affinity resin (Sigma Corp.), and yielded about 1.2 mg of pure DNA-PK. This enzyme was frozen in small aliquots and used for studies of *in vitro* phosphorylation of recombinant HDH II/Ku.

4.2.10. Characteristics of Purified DNA-PK

During the course of its purification, the enzymatic activity of DNA-PK was monitored by means of the kinase assay as described in materials and methods, in the presence of poly[dI-dC].poly[dI-dC], using dephosphorylated β casein as substrate (figure 4.2.9). Studies on the time course for the kinase reaction using this substrate showed that maximal phosphate transfer is obtained in the presence of 100 ng of poly[dI-dC].poly[dI-dC], and reaches a plateau after 15 minutes at 37° C (figure 4.2.10).

4.2.11. Phosphorylation of Recombinant HDH II/Ku by DNA-PK

To determine whether recombinant Ku heterodimer could serve as a substrate for DNA-PK, 2.5µg of the recombinant heterodimer were incubated, in the presence of γ -³²P ATP and 50 µM cold ATP, and in the presence or absence of DNA, with increasing amounts of the kinase. As is shown in figure 4.2.11, phosphorylation of both subunits of HDH II/Ku is DNA dependent and maximal incorporation of phosphate (a plateau) is obtained with 250 ng of DNA-PK.

Under these assay conditions, recombinant HDH II/Ku was phosphorylated with the kinase, in the presence 100 µM of cold ATP, and the resultant mixture was tested for DNA helicase activity.

4.2.12. The Helicase Activity of HDH II/Ku is Stimulated upon Phosphorylation by DNA-PK

100 ng of recombinant HDH II/Ku were phosphorylated with 250 ng of DNA-PK in the presence of 100 µM ATP and subsequently used in DNA unwinding assays alongside adequate controls consisting of HDH/Ku treated in the same manner but without DNA-PK, and DNA-PK alone. Under these assay conditions I observed a five fold stimulation of the helicase activity of phosphorylated HDH II/Ku with respect to the non-phosphorylated species (figure 4.2.12: compare lane 4 with lane 7; lane 5 with lane 8; lane 6 with lane 10), while DNA-PK alone showed no detectable DNA unwinding activity (lanes 2 and 3).

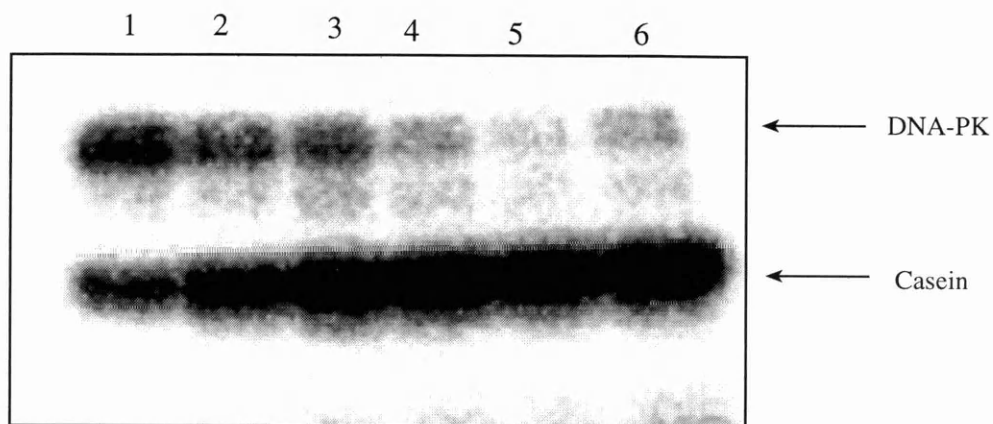


Figure 4.2.9. **Phosphorylation of β -Casein with DNA-PK in the presence of increasing amounts of DNA:** 800ng of casein were phosphorylated with 200 ng of DNA-Pk in the presence of 10 ng, 20 ng, 50 ng, 100 ng, 200 ng and 500 ng of poly dI-dC. poly dI-dC (lanes 1-6). As is shown in this figure, maximum phosphorylation is obtained with 100 ng of poly dI-dC. poly dI-dC. This kinase assay was used to monitor the activity of DNA-PK during the course of its purification.

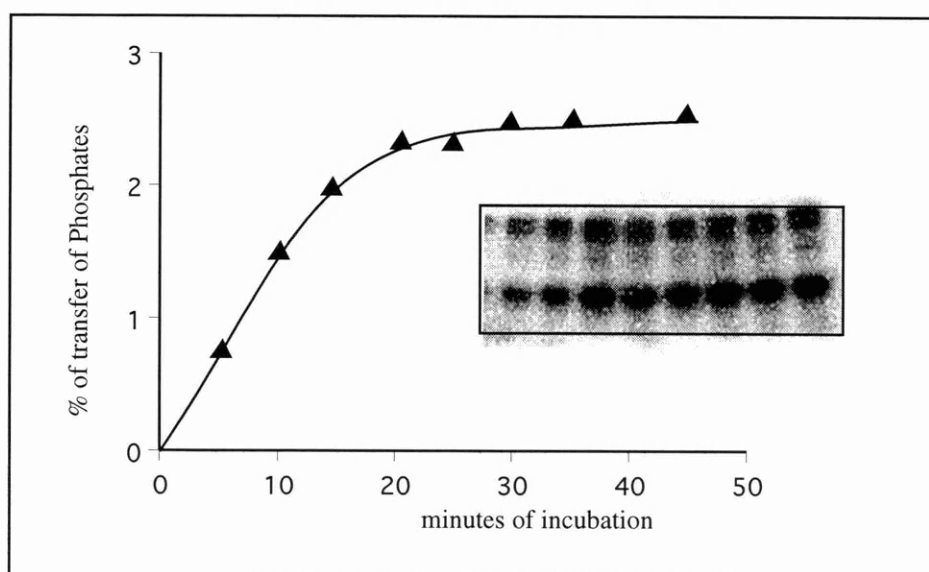


Figure 4.2.10. **Time Course of Phosphate Transfer to Casein by DNA-PK:** Time course study of the kinase reaction showed that the transfer of phosphate reached a plateau after 15 minutes and in the presence of 100ng of effector DNA. Inset is the autoradiography of the assay.

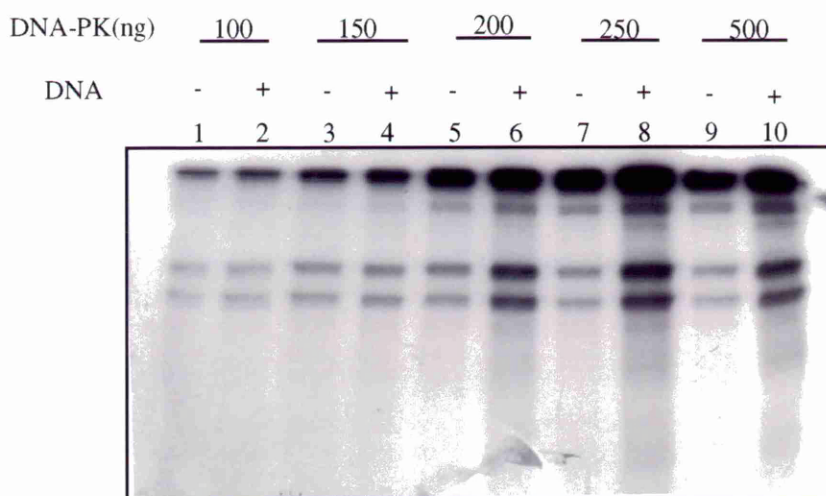
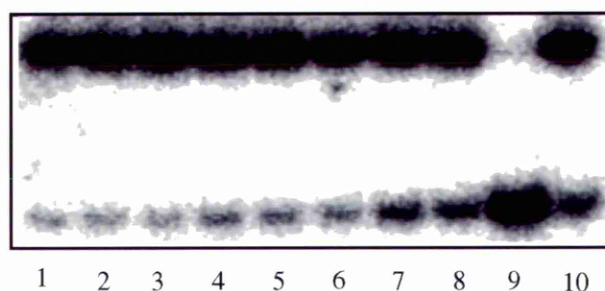


Figure 4.2.11. Phosphorylation of Recombinant HDH II/Ku by Increasing Amounts of DNA-PK: Recombinant HDH II/Ku heterodimer was phosphorylated with increasing amounts of DNA-Pk in the presence (+) or absence (-) of DNA. The amount of kinase used were: 100ng (lanes 1 and 2); 150 ng (lanes 3 and 4); 200 ng (lanes 5 and 6); 250 ng (lanes 7 and 8) and 500 ng (lanes 9 and 10).

A



B

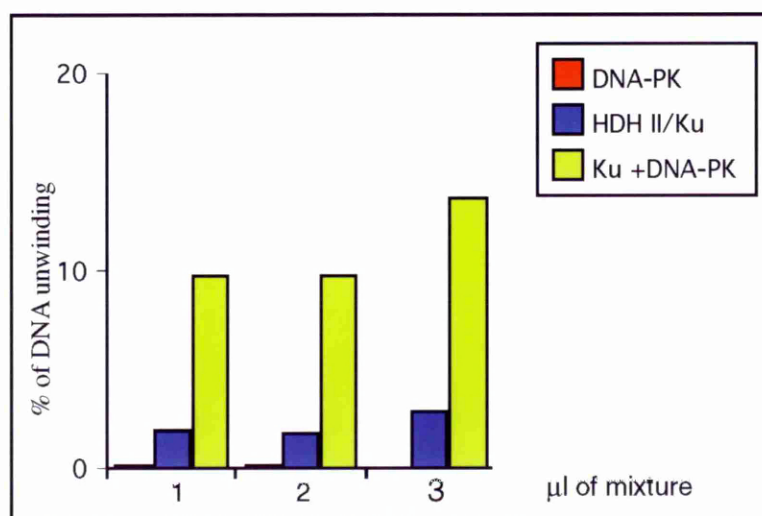


Figure 4.2.12. Effect of Phosphorylation on the Helicase Activity of HDH II/Ku: DNA unwinding was quantitated with non-phosphorylated as well as DNA-PK-phosphorylated HDH II/Ku in the presence of the standard helicase substrate. Panel A: lane 1, helicase substrate alone; lane 2, substrate with 1µl of DNA-PK alone; lane3, substrate with 1µl of mixture containing DNA-PK without HDH II; lanes 4 to 6, 1µl, 2µl, 3µl of non-phosphorylated HDH II respectively; lanes 7, 8 and 10, 1µl, 2µl, 3µl of phosphorylated HDH II/Ku respectively; lane 9 is heat denatured substrate.

Panel B: A graph representation shows more clearly the stimulation of the helicase activity of HDH II/Ku upon phosphorylation by DNA-PK.

5. DISCUSSION

In this thesis, I have described the biochemical properties of two different DNA unwinding enzymes isolated from HeLa cells namely, those of a novel DNA helicase showing a very high specific activity and species specific stimulation by RPA, HDH VII, and those of the separate subunits of the heterodimeric molecule, HDH II whose DNA binding and unwinding properties, in the heterodimeric form, had been described earlier.

Some of the biochemical properties of HDH VII, namely its very high specific activity, its strong but non-processive species-specific stimulation by hRPA and, above all, its double polarity of translocation, clearly distinguish it from all the other described eukaryotic DNA helicases.

An enzyme that moves in both directions along the DNA, must possess two structurally different but inter-modulating DNA binding domains on its molecule, each specific for either direction of translocation. On the single stranded portion of a substrate, each enzyme molecule can presumably bind with either binding domain and move accordingly, so that the overall enzyme movement results bi-directional. It is important that each DNA binding site of the enzyme modulates the DNA binding ability of the other in order to guarantee enzyme movement, which would otherwise be null, on the DNA molecule. The only other described DNA helicase operating with a double polarity of movement, namely the bacterial Rec BCD complex, playing a key role in the recombination pathway, is not stimulated by the SSB, it is highly processive, and can operate effectively on blunt-ended structures, all features that distinguish it markedly from HDH VII (168). Although the human analogue of an *E.coli* recombination helicase, the RecQL, co-purified with HDH VII through the initial chromatographic steps, this fortuitous event may not have any functional significance since both helicases were separated on a heparin sepharous resin as confirmed by the failure of a RecQL polyclonal antibody to recognize HDH VII in Western blotting (see figure 4.1.14)

Other properties of HDH VII, deducible from the data shown in figure 4.1.8, clearly indicate that the enzyme, in order to exert its maximal activity *in vitro*, requires a substrate conformation in which a short oligonucleotide is annealed to a longer filament, regardless of the presence or absence of hanging tails on the oligonucleotide.

On this kind of substrate, a comparison of the specific activity recorded for HDH VII with those observed for the other similar enzymes purified in our laboratory as well as that of the mouse FM3 helicase described by Hughes and Baldacci (139) (table 4.1.IV), showed that only HDH V possessed a specific activity comparable to that of HDH VII. However, the size of HDH V (92 kDa) and its uniquely 3' to 5' polarity of movement,

together with other biochemical features such as its susceptibility to increased ATP and salt concentrations, clearly distinguish this enzyme from HDH VII.

The major obstacle in defining *in vivo* roles for isolated eukaryotic DNA helicases is the lack of adequate *in vitro* assay systems that reproduce conditions in the cell since these enzymes usually function as components of multi-protein complexes *in vivo* (39-41). In this context, the strong and specific stimulation of the activity of HDH VII by hRPA does not offer a clear solution, since, in prokaryotes, a qualitatively similar stimulation is observed for unwinding enzymes involved in replication (191-193), repair and recombination (194). However, some features of this stimulation, namely its species-specificity and non-processivity, may offer some useful hints. It has been shown that various SSBs stimulate the processivity of the unwinding action of the SV40 large tumour antigen during viral DNA replication, but also that only hRPA may exert this stimulatory effect on the helicase during its initial bi-directional unwinding and activation of the origin. This specific stimulation of SV40 T antigen by hRPA resembles the same specific stimulation of HDH VII by the same SSB, suggesting a probable functional similarity of HDH VII for T antigen in the context of human DNA replication. Obviously replication in human cells is a much more complex process than replication in a virus due to the greater complexity of the system, and as such, no one single human protein would accomplish, in humans, the functions of the T antigen in viral DNA replication. But each component of a multi-enzyme complex involved in DNA replication in humans may mimic a specific aspect of the multiple functions of the T antigen and, interacting one with another, activate replication origins in higher eukaryotes.

The fact that the stimulation by hRPA fails to influence the processivity of HDH VII clearly indicates a molecule designed to accomplish a defined task after which it simply ceases to function, probably due to modifications on the substrate or on the enzyme itself. In the section of this thesis on "Results", I proposed two possible alternative mechanisms by which hRPA stimulated the DNA unwinding activity of HDH VII, namely, a transient interaction between the helicase and the SSB or alternatively that hRPA stimulated the helicase by acting exclusively at the DNA level. The observed failure of RPA to influence the processivity of HDH VII even in the presence of higher concentrations of the helicase or that of the SSB strongly supports the second alternative for the following reason: if the interaction between the two proteins were of a transient nature due, probably to the relatively low amount of one protein with respect to the other, then, in an experiment titrating one enzyme against a fixed concentration of the other, an optimal protein/protein ratio would have been attained to guarantee the formation of more stable complexes between the helicase and hRPA. The results of the time course of DNA unwinding by HDH VII indicates an enzyme that possesses a high rate of DNA unwinding. Therefore, if HDH VII formed even a transient complex with hRPA, such a hypothetical complex would have lived long enough to influence the processivity of this helicase given its high rate of DNA unwinding. Consequently, it was obvious that the human single stranded DNA binding protein, hRPA, stimulated the DNA

unwinding activity of HDH VII by acting at the DNA level through a mechanism which involved the stabilization of the unwound DNA single strands.

This ability of HDH VII to unwind only a short sequence in an RPA-stimulated fashion, suggested an analogy with the limited unwinding activity associated with the MCM group of proteins, a eukaryotic multi-protein complex widely documented as components of the eukaryotic replication initiation complex. In particular this low-processive helicase activity of the MCMs is associated with the licensing of DNA replication, a process believed to involve two component factors: RLF-M, composed of the MCM group of proteins and RLF-B, a hitherto unidentified enzyme whose activity should modulate the activity of RLF-M (144,195). The special functions of RLF-B would require that an enzyme possessed dynamic DNA interaction properties characterized by an active DNA binding state, an inactive state that doesn't bind DNA and possible intermediate states. Such properties are similar to the observed binding/interactions of HDH VII with DNA in the course of its purification. Infact during five different preparations of HDH VII from asynchronously growing, *in vitro* cultured HeLa cells I observed variations in the specific activity of the pure enzyme from a value 1.8×10^7 to that of 4×10^7 U/mg of enzyme, and these variations were parallel with similar variations in the molar range of salt used to elute the enzyme from a ssDNA-cellulose column. Since average protein recovery, for this enzyme, was constant in these different purifications, the observed variations in the specific activity may only be reasonably explained by variations in the DNA-interaction state of the enzyme, all of which are intermediate states between the totally active and totally inactive state of DNA binding. All these observed properties of HDH VII positively indicate it as an important human protein involved in a fundamental DNA process *in vivo*.

Although attempts at microsequencing have so far been unsuccessful, data on the mass comparison is quite encouraging and certainly indicate that HDH VII is a novel human protein since the mass of the fragments of the tryptic digestion do not match with the mass of similarly generated fragments of any known human protein. Efforts are in progress to isolate substantial amounts of this protein from a larger amount of cultured cells to guarantee the determination of the amino acid sequence.

Finally, the estimated abundance of the enzyme (between 30 000 and 50 000 molecules per nucleus) is consistent with a possible function in initial origin opening, considering the estimated number of approximately 30 000 replicons per human genome. Since eukaryotic DNA replication origins fire asynchronously, the catalytic properties and the limited processivity of HDH VII also argue for an enzyme that could possibly participate in activating an origin thereafter dissociating, once the origin is active, to migrate to another unactivated origin of replication. Only future work aimed at identifying the gene(s) codifying it, may offer further clues and tools for the unambiguous identification of the *in vivo* function of this enzyme.

By virtue of its *in vitro* biochemical properties, HDH VII is clearly an enzyme uniquely involved in a limited unwinding of partially duplex DNA molecules in an RPA-stimulated manner. The same may not be said of the heterodimeric molecule HDH II/Ku, an enzyme endowed with both dsDNA binding and unwinding activities *in vitro*, and which has been shown to be involved in V(D)J recombination as well as the repair of DNA double strand breaks. The differences observed in the *in vitro* properties of the two enzymes described in this thesis further stress the notion that different helicases are involved in different DNA metabolic processes *in vivo*.

The results obtained in the experiments presented in the relative sections of this thesis show the possibility of uncoupling these two *in vitro* activities of the HDH II/Ku heterodimer. The properties of the refolded HDH II/Ku and of the two separate subunits show that the DNA unwinding activity ascribed to the heterodimer resides in fact exclusively in the 70 kDa subunit. The DNA end-binding capacity, however, remains a prerogative of the HDH II/Ku heterodimer, suggesting that the presence of this molecular form is required to perform the reactions necessary for repair of double strand breaks and V(D)J recombination. Whether this scenario represents the situation *in vivo* is hard to say from this study but certainly raises the speculation of a possible differential *in vivo* expression of these subunits according to the circumstantial cellular requirements for Ku. It should be remembered that the genes for the Ku subunits are localized on different chromosomes (196) and that a number of reports have attributed different properties to either subunit of Ku, suggesting that the enzyme may not always exist in its heterodimeric form (197,198). Since the heterodimer has always been isolated from proliferating nuclei not much is known about the nature and structure of this protein in other phases of the cell cycle. Furthermore, the 70 kDa subunit alone has been immunologically localized to the nucleoli and periphery of interphase nuclei (199). The presence of helicase activity in the 70 kDa subunit alone offers additional circumstantial evidence of the possible independent *in vivo* existence of this subunit in certain conditions and of possible additional roles for this molecule in DNA metabolism.

The observation that the unwinding activity resides in the small subunit whereas the heterodimeric structure is required for duplex DNA end-binding, confirms our previous inference (based on the lack of reciprocal inhibition of either activity by the substrate of the other one) that these two properties are located in two different moieties of the Ku heterodimer (116).

Evaluation of the DNA unwinding at different substrate concentrations gave a K_m value of 0.5 nM for the three active species of HDH II/Ku. Thus, the three species have the same affinity for the helicase substrate notwithstanding their relative conformational and supramolecular differences. The differences in the observed V_{max} values are parallel with the

different affinities for ATP, a not surprising observation, considering the fact that DNA unwinding by helicases strictly depends on ATP hydrolysis.

The fact that the ATPase activity of the 83 kDa subunit is not associated to any appreciable helicase activity leaves unexplained its possible functional significance, although similar cases have been reported (200). It is conceivable that, in the heterodimeric form, the intrinsic ATPase function of the 83 kDa subunit may be the basis for the higher V_{\max} of the heterodimer as a helicase (more than 4 fold) with respect to the 70 kDa subunit alone. The likely absence of a helicase active centre in the 83 kDa subunit makes its ssDNA-dependent ATPase activity a futile one, at least at first sight.

In contrast to the situation with the unwinding activity, the DNA binding capacity was restored by the renaturation procedure rather poorly also for the heterodimer. In fact, in different preparations, we have observed a significant variability in the extent of reconstitution of DNA end-binding capacity of Ku, reaching in some case 20% of the activity of the native Ku heterodimer purified from HeLa cells. Furthermore, this capacity also proved very labile upon storage at 4°C, much more than for native Ku. The reasons for this discrepancy (with respect to the satisfactory reconstitution of unwinding activity) may depend on the fact that: 1) the helicase active centre (and the ssDNA binding moiety) resides on a well distinct position of the molecule with respect to the duplex DNA end-binding domain; 2) post-translational modifications of Ku operating *in vivo* may enhance and stabilize the duplex end-binding capacity.

The separate subunits showed only minimal or no duplex DNA binding ability, even with respect to the reduced reconstituted property of the heterodimer; in fact, only the small subunit showed a binding capacity of the order of 1% of the reconstituted recombinant dimer. From my data it appears that the formation of a dimeric structure *per se* is not enough to confer the property of DNA binding, since the large subunit can form homodimers without reacquiring the ability to bind DNA. It appears therefore that the duplex DNA binding ability is an intrinsic property of a specific molecular environment produced by the formation of the heterodimer.

This observation may appear in contrast with previous reports from different laboratories that the separate subunits show ability to bind DNA probes in a South-Western type of assay, the 70 kDa subunit having greater affinity than the large one (201). On the other hand, the assay for electrophoretic gel retardation is a much more stringent one than the South-Western, and it is likely that this discrepancy is purely apparent. In fact, if the interaction of the small subunit with DNA is also measured (see e.g. Wang et al. (202) with other methods that, like the South-Western assay, do not allow a precise determination of the K_d value, this molecule indeed shows a certain capacity of binding duplex DNA. The same authors, on the other hand, confirmed that the 70 kDa subunit does not cause any appreciable band shift on duplex DNA.

Wu and Lieber (203), by a completely different approach (two hybrid and biochemical analyses of the interactions of Ku 70 and 83 and of truncated forms of these molecules) in fact arrived to the same conclusions as this report: in their work, neither the 70 nor the 83 kDa subunit, when translated alone, was able to bind DNA in band-shift assays, whereas they did so when translated jointly. These authors were also able to ascribe to the C-terminal regions of the two subunits the properties of heterodimer formation and DNA binding. It seems reasonable to conclude at this stage that neither subunit alone can bind duplex DNA efficiently.

These considerations do not apply to the ability to bind ssDNA, which is in all probability related (functionally and physically) to the unwinding capacity of Ku. In fact, the affinities of the recombinant heterodimer and of the 70-kDa subunit for ssDNA are quantitatively comparable to the reconstituted DNA unwinding capacity of the same molecular forms (see Tables 4.2.I and 4.2.II). Also, the 83-kDa subunit homodimer maintains a trace (small but significant) of ssDNA affinity, that probably sustains the trace of ssDNA-dependent ATPase activity observed for this molecular form.

Structural data on Ku are not yet available, but the properties measured by biochemical means would favour the presence of some form of ring structure in the heterodimer, able to bind to ends, and then slide along the duplex DNA in a nearly irreversible way. This observation is in agreement with the wealth of data indicating a strong but promiscuous requirement of Ku for binding DNA ends, since the heterodimer can bind them irrespectively of their DNA sequence or their chemical detailed structure (5' protruding, 3' protruding, phosphorylated or non-phosphorylated, hairpin, etc.). This contention is further strengthened by the demonstrated inability of Ku antigen to bind to circular duplexes and by the inability of Ku bound to a duplex to be removed when the loaded duplex is closed into a circle (204). In contrast to this observation are the results reported by Giffin et al. (205,206) of a sequence specificity for Ku, that apparently allows the binding of the molecule to a circular DNA duplex. Whereas this binding (whose mechanism remains to be described) might be related to the proposed function of Ku as a transcription factor (206), the most popular model for the Ku interaction with the DNA duplex remains that of binding at the end and sliding along the duplex to form "beads on a string" with a 25 bp periodicity. The ability of Ku to bind frayed ends would fit very well with the function of Ku in the site-specific recombination processes required for immunoglobulin gene maturation and for X-ray damage repair.

This observation is further supported by the recent results obtained by Ramsden et al (207) who found evidence of a direct role for the Ku protein in the repair of double strand DNA breaks. These authors reported that under optimal conditions of salt, 120 mM, and temperature, 37°C, Ku specifically stimulated the ligation of DNA fragments bearing short

(or none) bp overhangs by eukaryotic DNA ligases. Thus in this case as well, the function of the Ku protein was that of holding together two free dsDNA termini for the sealing action of the ligase to occur.

Obviously, the presence of a helicase activity (active only on DNA partial duplexes, and not on RNA containing structures) could also fit very well with these events, even though no evidence has been available so far in this sense. The production of mutations and deletions in either subunit and the study of the functional effects of these mutations on those cellular processes will shed more light in the future on the *in vivo* mode of action of this important molecule, and on its possible involvement in other cellular processes.

I report the successful separation of the components of the DNA-dependent protein kinase complex and the stimulation of the helicase activity of recombinant HDH II/Ku upon phosphorylation by this kinase. This increase in the helicase activity of phosphorylated HDH II suggests a tight relationship between the phosphorylation-state of the enzyme and its DNA unwinding activity. Since I have also observed that the various species of HDH II exhibited the same affinity for the helicase substrate but show different affinities for ATP, the observed increase in the DNA unwinding activity of recombinant HDH II upon phosphorylation may best be explained by a corresponding increase in the affinity of this enzyme species for ATP which would give rise to increased hydrolysis of the co-factor and consequent increase in DNA unwinding. I am currently investigating the possibility of phosphorylating the separate subunits of HDH II with DNA-PK (DNA binding for these separate subunits is hardly detectable) and the effects such modification may have on the properties described here for these separate subunits. I have, as yet, not explored the possible effects of phosphorylation on the DNA end-binding activity of HDH II/Ku due to the inherent conflicts between the methodologies of the protein kinase assay and the DNA gel-retardation assay. HDH II is phosphorylated by the kinase in the presence of 100 ng of poly[dI-dC].poly[dI-dC] used as DNA co-factor, and any gel shift experiment with this mixture would imply a very large excess, in weight, of the poly[dI-dC].poly[dI-dC] with respect to the few picograms of the gel shift probe. I estimate that this excess would lie between 1000 and 10000 fold, and earlier results obtained in this laboratory show that such excess is sufficient to abolish DNA binding and gel shifting by HDH II/Ku. I am, therefore, optimizing a technique to separate the components of the protein-kinase mixture to render the DNA gel retardation assay with phosphorylated HDH II/Ku feasible.

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